

U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER LIDER1
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		U.S. APPLICATION NO. (If known, see 37 CFR 1.5) 09/763293
INTERNATIONAL APPLICATION NO. PCT/IL99/00448	INTERNATIONAL FILING DATE 19 August 1999	PRIORITY CLAIMED 21 August 1998
TITLE OF INVENTION ANTI-INFLAMMATORY PEPTIDES DERIVED FROM IL-2 AND ANALOGUES THEREOF		
APPLICANT(S) FOR DO/EO/US LIDER, Ofer et al.		

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☒ The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☐ is attached hereto (required only if not transmitted by the International Bureau).
 - b. ☒ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. ☐ An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A **FIRST** preliminary amendment.
 ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
 - ☒ Courtesy copy of the International Application as filed.
 - ☒ Courtesy copy of the first page of the International Publication (WO 00/11028).
 - ☒ Courtesy copy of the International Preliminary Examination Report. There were no annexes.
 - ☒ Formal drawings, 8 sheets, Figures 1-8E.
 - ☒ Courtesy Copy of the International Search Report
 - ☒ Applicant claims small entity status. See 37 CFR 1.27.
 - ☒ Paper copy of sequence listing.

U.S. APPLICATION NO. (if known, see 37 CFR 1.5) <div style="font-size: 24pt; font-weight: bold;">09/763293</div>		International Application No PCT/IL99/00448		Attorney's Docket No LIDER1		
<div>17. [xx] The following fees are submitted:</div> <div>BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) –(5): Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....\$1000.00</div> <div>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....\$860.00</div> <div>International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....\$710.00</div> <div>International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....\$690.00</div> <div>International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....\$100.00</div> <div style="text-align: right; margin-top: 10px;">ENTER APPROPRIATE BASIC FEE AMOUNT =</div>				CALCULATIONS PTO USE ONLY		
ENTER APPROPRIATE BASIC FEE AMOUNT =				\$ 860.00		
Surcharge of \$130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).				\$ 130.00		
Claims as Originally Presented		Number Filed	Number Extra	Rate		
Total Claims		14 - 20	0	X \$18.00	\$ 0	
Independent Claims		2 - 3	0	X \$80.00	\$ 0	
Multiple Dependent Claims (if applicable)				+\$270.00	\$ 0	
TOTAL OF ABOVE CALCULATIONS =				\$ 990.00		
Claims After Post Filing Prel. Amend		Number Filed	Number Extra	Rate		
Total Claims		- 20		X \$18.00	\$ 0	
Independent Claims		- 3		X \$78.00	\$ 0	
TOTAL OF ABOVE CALCULATIONS =				\$ 990.00		
Reduction of ½ for filing by small entity, if applicable. Applicant claims small entity status. See 37 CFR 1.27.				\$ 495.00		
SUBTOTAL =				\$ 495.00		
Processing fee of \$130.00 for furnishing the English translation later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$		
TOTAL NATIONAL FEE =				\$ 495.00		
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$		
TOTAL FEES ENCLOSED =				\$ 495.00		
					Amount to be:	\$
					refunded	
					charged	\$
a. [] A check in the amount of \$ _____ to cover the above fees is enclosed.						
b. [XX] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of <u>\$495.00</u> , is attached.						
c. [] Please charge my Deposit Account No. <u>02-4035</u> in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.						
d. [XX] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>02-4035</u> . A duplicate copy of this sheet is enclosed.						
NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.						
SEND ALL CORRESPONDENCE TO:						
BROWDY AND NEIMARK, P.L.L.C.						
624 NINTH STREET, N.W., SUITE 300						
WASHINGTON, D.C. 20001						
TEL: (202) 628-5197						
FAX: (202) 737-3528						
Date of this submission: February 21, 2001						
					<div style="font-size: 18pt; font-family: cursive;">Roger L. Browdy</div> <div>SIGNATURE</div>	
					<div>_____ Roger L. Browdy</div> <div>NAME</div>	
					<div><u>25.618</u></div> <div>REGISTRATION NUMBER</div>	

09/763293

JCO2 Rec'd PCT/PTO 21 FEB 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

ATTY.'S DOCKET: LIDER1

In re Application of:)	Art Unit:
O. LIDER et al.)	
)	Examiner:
I.A. No. PCT/IL99/00448)	
Appln. No.: not yet assigned)	Washington, D.C.
)	
I.A. Filed: 19 August 1999)	
Filed: even date herewith)	February 21, 2001
)	
For: ANTI-INFLAMMATORY...)	
)	

PRELIMINARY AMENDMENT

Honorable Commissioner for Patents
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and
prior to calculation of the filing fee, kindly amend as
follows:

IN THE SPECIFICATION

After the title please insert the following
paragraph:

REFERENCE TO RELATED APPLICATIONS

The present application is the national stage under
35 U.S.C. 371 of international application PCT/IL99/00448,
filed 19 August 1999, which designated the United States,
which international application was published under PCT
Article 21(2) in English.

IN THE CLAIMS

Please delete claims 13 and 15-17.

In re Appln. of O. LIDER et al. (LIDER1)

Claim 3, line 1, delete "or 2".

Claim 4, line 1, change "any one of claims 1 to 3"
to --claim 1--.

Claim 14, line 2, change "any one of claims 1 to 10"
to --claim 1--.

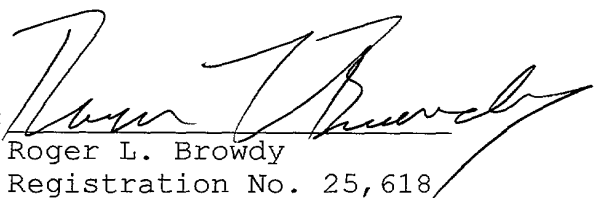
Claim 18, line 3, change "any one of claims 1 to 10"
to --claim 1--.

REMARKS

The above amendment to the specification is being made to insert reference to the PCT application of which the present case is a U.S. national stage. The above amendments to the claims are being made in order to eliminate any properly multiply dependent claims, for the purpose of reducing the filing fee. Please enter this amendment prior to calculation of the filing fee in this case.

Favorable consideration and allowance are earnestly solicited.

Respectfully submitted,
BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant

By: 
Roger L. Browdy
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09/23/99 05:10:50

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7 MAY 2001

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:)	Box PCT
)	
LIDER et al)	Examiner:
)	
Appln. No.: 09/763,293)	Washington, D.C.
)	
Filed: not yet received)	
)	
IA No. PCT/IL99/00448)	
IA Filed: 19 August 1999)	May 29, 2001
)	
For: ANTI-INFLAMMATORY)	Atty.Docket: LIDER=1
PEPTIDES DERIVED FROM IL-2))	
AND ANALOGUES THEREOF)	

RESPONSE TO NOTIFICATION TO COMPLY WITH
SEQUENCE LISTING REQUIREMENTS

Honorable Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notification to Comply dated
March 29, 2001, and prior to the examination of the above-
described application, please amend the present application as
follows:

IN THE SPECIFICATION

Please substitute the attached Sequence Listing
section, pages 1-10, for those previously submitted.

Please substitute the attached specification for the
one previously submitted. A copy of the originally filed
specification with the changes marked in revision mode is also
attached.

10 10 10 10 10

IN THE CLAIMS

Claims 4 and 6-10 have been amended as follows:

4 (Amended). A synthetic peptide according to any one of claim 1, selected from:

(i) peptides **pep1**, **pep2**, and **pep3** of the sequences:

(**pep1**) Ile-Val-Leu

(**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)

(**pep3**) Arg-Met-Leu-Thr (SEQ ID NO:2)

(ii) peptides obtained from **pep2** by deletion of one or more amino acid residues;

(iii) peptides obtained by addition to peptides (i) or (ii) of one or more natural or non-natural amino acid residues;

(iv) peptides obtained by replacement of one or more amino acid residues of peptides (i) to (iii) by the corresponding D-stereomer, by another natural amino acid residue or by a non-natural amino acid residue;

(v) chemical derivatives of the peptides (i) to (iv);

(vi) cyclic derivatives of peptides (i) to (v);

(vii) dual peptides consisting of two of the same or different peptides (i) to (vi), wherein the peptides are covalently linked to one another directly or through a spacer; and

(viii) multimers comprising a number of the same or different peptides (i) to (vi).

6 (Amended). A synthetic peptide according to claim 5, selected from:

(**pep1**) Ile-Val-Leu
(**pep4**) Asn-Ile-Asn-Val-Ile-Val-Leu (SEQ ID NO:3),
(**pep5**) Ile-Val-Leu-Glu-Leu-Lys-Gly (SEQ ID NO:4),
(**pep6**) Asn-Val-Ile-Val-Leu (SEQ ID NO:5)
(**pep7**) Ala-Val-Leu
(**pep8**) Ile-Ala-Leu
(**pep9**) Ile-Val-Ala
(**pep10**) Glu-Val-Leu

(**pep11**, linear) and (**pep12**, cyclic) Cys-Ile-Val-Leu-Ala-Cys (SEQ ID NO:6) and,

(**pep13**, linear) and (**pep14**, cyclic) Cys-Ile-Val-Leu-Ala-Ala-Cys (SEQ ID NO:7).

7 (Amended). The synthetic peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) (**pep2**), and derivatives thereof according to claim 4, obtained by:

(a) elongation by up to 4 further amino acid residues at the C and/or N terminal ends, preferably according to the natural sequence of IL-2;

(b) substitution of the Glu residue by a natural or non-natural charged or polar charged amino acid residue, preferably selected from Lys, Arg, Asp, Gln, Asn;

(c) substitution of the Phe residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(d) substitution of the Leu residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid

residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(e) substitution of the important Asn residue by a hydrophilic, non-charged, aliphatic natural or non-natural amino acid residue such as Gln;

(f) substitution of the Arg residue by a positively charged, natural or non-natural amino acid residue, preferably selected from Lys, Orn, homoArg;

(g) substitution of the Trp residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Ile, Leu, Nle, Tic, Phe, 4-phenyl-Phe, 4-methyl-Phe;

(h) substitution of the Ile residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Phe, Leu, Nle, Tic;

(i) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue preferably selected from Cys, Ser;

(j) truncation by up to 4 amino acid residues from either the C or N terminal;

(k) amidation of the C-terminal Thr;

(l) cyclization of **pep2** or of any peptide of (a) to (k); and

(m) any combination of (a) to (l).

8 (Amended). A peptide according to claim 7, selected from:

(**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)

(**pep15**) Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:8)

(**pep16**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys (SEQ ID NO:9)

(**pep17**) Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:10)

(**pep18**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser (SEQ ID NO:11)

(**pep19**) Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:12)

(**pep20**) Arg-Trp-Ile-Thr (SEQ ID NO:13)

(**pep21**) Glu-Phe-Leu-Asn (SEQ ID NO:14)

(**pep22**) Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:15)

(**pep23**) Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:16)

(**pep24**) Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:17)

(**pep25**) Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:18)

(**pep26**) Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:19)

(**pep27**) Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr (SEQ ID NO:20)

(**pep28**) Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr (SEQ ID NO:21)

(**pep29**) Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr (SEQ ID NO:22)

(**pep30**) Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr (SEQ ID NO:23)

(**pep31**) Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr (SEQ ID NO:24)

(**pep32**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala (SEQ ID NO:25)

(**pep33**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-NH₂ (SEQ ID NO:26) and,

(**pep34**, linear) and (**pep35**, cyclic) Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys (SEQ ID NO:27).

9 (Amended). The synthetic peptide Arg-Met-Leu-Thr (SEQ ID NO:2) (**pep3**), and derivatives thereof according to claim 4, obtained by:

(a) elongation by up to 4 further amino acid residues at the C and/or N terminal end, preferably according to the natural sequence of IL-2;

(b) substitution of the Arg residue by a natural or non-natural positively charged amino acid residue, preferably selected from Lys, Orn, homoArg, diaminobutyric acid;

(c) substitution of the Met residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Ile, Leu, Nle, Tic;

(d) substitution of the Leu residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Nle, Tic;

(e) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue such as Ser, Cys;

(f) amidation of the C-terminal Thr residue;

(g) cyclization of **pep3** or of any peptide of (a) to (f); and

(h) any combination of (a) to (g).

10 (Amended). A peptide according to claim 9, selected from:

(**pep3**) Arg-Met-Leu-Thr (SEQ ID NO:2)

(**pep36**) Ala-Met-Leu-Thr (SEQ ID NO:28)

(**pep37**) Arg-Ala-Leu-Thr (SEQ ID NO:29)

(**pep38**) Arg-Met-Ala-Thr (SEQ ID NO:30)

(**pep39**) Arg-Met-Leu-Ala (SEQ ID NO:31)

(**pep40**) Lys-Met-Leu-Thr (SEQ ID NO:32)

(**pep41**) Arg-Val-Leu-Thr (SEQ ID NO:33)

(**pep42**) Arg-Met-Leu-Thr-NH₂ (SEQ ID NO:34)

(**pep43**) Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr (SEQ ID NO:35)

(**pep44**) Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr (SEQ ID NO:36) and,

(**pep45**, linear) and (**pep46**, cyclic) Cys-Arg-Met-Leu-Thr-Ala-Cys (SEQ ID NO:37).

In re Appln. No. 09/763,293

IN THE ABSTRACT

A new Abstract of the Disclosure on a separate sheet to replace the Abstract of the Disclosure as originally filed is attached.

09/763,293 05/13/04

REMARKS

Applicants have entered into the present specification a substitute paper copy Sequence Listing section according to 37 C.F.R. §1.821(c) as new pages 1-10. The substitute Sequence Listing section contains clerical corrections in sections <110>, <130>, <140, 141>, and <150, 151> and in sections <223> of the sequences. Furthermore, attached hereto is a 3 1/2" disk containing the "Sequence Listing" in computer readable form in accordance with 37 C.F.R. §1.821(e).

Applicants have amended the specification to insert SEQ ID Nos, as supported in the present specification.

The following statement is provided to meet the requirements of 37 C.F.R. §1.825(a) and 1.825(b).

I hereby state, in accordance with 37 C.F.R. §1.825(a), that the amendments included in the substitute sheets of the sequence listing are believed to be supported in the application as filed and that the substitute sheets of the sequence listing are not believed to include new matter.

I hereby further state, in accordance with 37 C.F.R. §1.825(b), that the attached copy of the computer readable form is the same as the attached substitute paper copy of the sequence listing.

Under U.S. rules, each sequence must be classified in <213> as an "Artificial Sequence", a sequence of "Unknown" origin, or a sequence originating in a particular organism, identified by its scientific name.

Neither the rules nor the MPEP clarify the nature of the relationship which must exist between a listed sequence and an organism for that organism to be identified as the origin of the sequence under <213>.

Hence, counsel may choose to identify a listed sequence as associated with a particular organism even though that sequence does not occur in nature by itself in that organism (it may be, e.g., an epitopic fragment of a naturally occurring protein, or a cDNA of a naturally occurring mRNA, or even a substitution mutant of a naturally occurring sequence). Hence, the identification of an organism in <213> should not be construed as an admission that the sequence *per se* occurs in nature in said organism.

Similarly, designation of a sequence as "artificial" should not be construed as a representation that the sequence has no association with any organism. For example, a primer or probe may be designated as "artificial" even though it is necessarily complementary to some target sequence, which may occur in nature. Or an "artificial" sequence may be a substitution mutant of a natural sequence, or a chimera of two or more natural sequences, or a cDNA (i.e., intron-free sequence) corresponding to an intron-containing gene, or otherwise a fragment of a natural sequence.

The Examiner should be able to judge the relationship of the enumerated sequences to natural sequences by giving full consideration to the specification, the art cited therein, any further art cited in an IDS, and the

results of his or her sequence search against a database containing known natural sequences.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version with markings to show changes made".

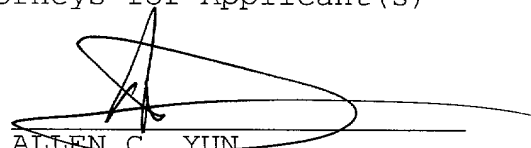
Applicants submit that the present application contains patentable subject matter and therefore urge the examiner to pass the case to issuance.

If the examiner has any questions or comments concerning the above described application, the examiner is urged to contact the undersigned at the phone number below.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

By


ALLEN C. YUN
Registration No. 37,971

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F:\,Y\YEDA\Lider1\pto\RESPONSE TO NOTICE TO COMPLY.doc

VERSION WITH MARKINGS TO SHOW CHANGES MADE

In the specification:

A substitute specification has been submitted.

In the claims:

Claims 4 and 6-10 have been amended as follows:

4 (Amended). A synthetic peptide according to any one of claim 1, selected from:

(i) peptides **pep1**, **pep2**, and **pep3** of the sequences:

(**pep1**) Ile-Val-Leu

(**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)

(**pep3**) Arg-Met-Leu-Thr (SEQ ID NO:2)

(ii) peptides obtained from **pep2** by deletion of one or more amino acid residues;

(iii) peptides obtained by addition to peptides (i) or (ii) of one or more natural or non-natural amino acid residues;

(iv) peptides obtained by replacement of one or more amino acid residues of peptides (i) to (iii) by the corresponding D-stereomer, by another natural amino acid residue or by a non-natural amino acid residue;

(v) chemical derivatives of the peptides (i) to (iv);

(vi) cyclic derivatives of peptides (i) to (v);

(vii) dual peptides consisting of two of the same or different peptides (i) to (vi), wherein the peptides are covalently linked to one another directly or through a spacer; and

(viii) multimers comprising a number of the same or different peptides (i) to (vi).

6_(Amended). A synthetic peptide according to claim 5, selected from:

(pep1) Ile-Val-Leu
(pep4) Asn-Ile-Asn-Val-Ile-Val-Leu (SEQ ID NO:3),
(pep5) Ile-Val-Leu-Glu-Leu-Lys-Gly (SEQ ID NO:4),
(pep6) Asn-Val-Ile-Val-Leu (SEQ ID NO:5)
(pep7) Ala-Val-Leu
(pep8) Ile-Ala-Leu
(pep9) Ile-Val-Ala
(pep10) Glu-Val-Leu
(pep11, linear) and (pep12, cyclic) Cys-Ile-Val-Leu-Ala-Cys (SEQ ID NO:6) and,
(pep13, linear) and (pep14, cyclic) Cys-Ile-Val-Leu-Ala-Ala-Cys (SEQ ID NO:7).

7_(Amended). The synthetic peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) (pep2), and derivatives thereof according to claim 4, obtained by:

(a) elongation by up to 4 further amino acid residues at the C and/or N terminal ends, preferably according to the natural sequence of IL-2;

(b) substitution of the Glu residue by a natural or non-natural charged or polar charged amino acid residue, preferably selected from Lys, Arg, Asp, Gln, Asn;

(c) substitution of the Phe residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid

residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(d) substitution of the Leu residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(e) substitution of the important Asn residue by a hydrophilic, non-charged, aliphatic natural or non-natural amino acid residue such as Gln;

(f) substitution of the Arg residue by a positively charged, natural or non-natural amino acid residue, preferably selected from Lys, Orn, homoArg;

(g) substitution of the Trp residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Ile, Leu, Nle, Tic, Phe, 4-phenyl-Phe, 4-methyl-Phe;

(h) substitution of the Ile residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Phe, Leu, Nle, Tic;

(i) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue preferably selected from Cys, Ser;

(j) truncation by up to 4 amino acid residues from either the C or N terminal;

(k) amidation of the C-terminal Thr;

(l) cyclization of **pep2** or of any peptide of (a) to (k); and

(m) any combination of (a) to (l).

8 (Amended). A peptide according to claim 7,
selected from:

(**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID
NO:1)

(**pep15**) Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr
(SEQ ID NO:8)

(**pep16**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys
(SEQ ID NO:9)

(**pep17**) Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-
Ile-Thr (SEQ ID NO:10)

(**pep18**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-
Gln-Ser (SEQ ID NO:11)

(**pep19**) Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:12)

(**pep20**) Arg-Trp-Ile-Thr (SEQ ID NO:13)

(**pep21**) Glu-Phe-Leu-Asn (SEQ ID NO:14)

(**pep22**) Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID
NO:15)

(**pep23**) Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID
NO:16)

(**pep24**) Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID
NO:17)

(**pep25**) Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID
NO:18)

(**pep26**) Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr (SEQ ID
NO:19)

(**pep27**) Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr (SEQ ID NO:20)

(**pep28**) Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr (SEQ ID NO:21)

(**pep29**) Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr (SEQ ID NO:22)

(**pep30**) Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr (SEQ ID NO:23)

(**pep31**) Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr (SEQ ID NO:24)

(**pep32**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala (SEQ ID NO:25)

(**pep33**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-NH₂ (SEQ ID NO:26) and,

(**pep34**, linear) and (**pep35**, cyclic) Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys (SEQ ID NO:27).

9 (Amended). The synthetic peptide Arg-Met-Leu-Thr (SEQ ID NO:2) (**pep3**), and derivatives thereof according to claim 4, obtained by:

(a) elongation by up to 4 further amino acid residues at the C and/or N terminal end, preferably according to the natural sequence of IL-2;

(b) substitution of the Arg residue by a natural or non-natural positively charged amino acid residue, preferably selected from Lys, Orn, homoArg, diaminobutyric acid;

(c) substitution of the Met residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid

residue, preferably selected from Phe, Tyr, Ile, Leu, Nle, Tic;

(d) substitution of the Leu residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Nle, Tic;

(e) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue such as Ser, Cys;

(f) amidation of the C-terminal Thr residue;

(g) cyclization of **pep3** or of any peptide of (a) to (f); and

(h) any combination of (a) to (g).

10 (Amended). A peptide according to claim 9, selected from:

(**pep3**) Arg-Met-Leu-Thr (SEQ ID NO:2)

(**pep36**) Ala-Met-Leu-Thr (SEQ ID NO:28)

(**pep37**) Arg-Ala-Leu-Thr (SEQ ID NO:29)

(**pep38**) Arg-Met-Ala-Thr (SEQ ID NO:30)

(**pep39**) Arg-Met-Leu-Ala (SEQ ID NO:31)

(**pep40**) Lys-Met-Leu-Thr (SEQ ID NO:32)

(**pep41**) Arg-Val-Leu-Thr (SEQ ID NO:33)

(**pep42**) Arg-Met-Leu-Thr-NH₂ (SEQ ID NO:34)

(**pep43**) Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr (SEQ ID NO:35)

(**pep44**) Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr (SEQ ID NO:36) and,

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(**pep45**, linear) and (**pep46**, cyclic) Cys-Arg-Met-
Leu-Thr-Ala-Cys (SEQ ID NO:37).

Figure 1 consists of 12 histograms arranged in a single row. Each histogram represents the frequency distribution of the number of non-zero elements in the vector x for a specific value of n . The x-axis for all histograms is 'Number of non-zero elements in x ' with major ticks at 0, 20, 40, 60, 80, 100, and 120. The y-axis is 'Frequency' with major ticks at 0, 2, 4, 6, 8, and 10. The histograms are labeled with n values: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120. As n increases, the distribution becomes more concentrated around n , and the peak frequency increases.

Figure 1 consists of 12 histograms arranged in a single row. Each histogram represents the frequency distribution of the number of non-zero elements in the vector x for a specific value of n . The x-axis for all histograms is 'Number of non-zero elements in x ' with major ticks at 0, 20, 40, 60, 80, 100, and 120. The y-axis is 'Frequency' with major ticks at 0, 2, 4, 6, 8, and 10. The histograms are labeled with n values: 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, and 120. As n increases, the distribution becomes more concentrated around n , and the peak frequency increases.

09/763293

ANTI-INFLAMMATORY PEPTIDES DERIVED FROM IL-2 AND ANALOGUES
THEREOF

REFERENCE TO RELATED APPLICATIONS

- 5 The present application is the national stage under 35 U.S.C. 371 of international application PCT/IL99/00448, filed 19 August 1999, which designated the United States, which international application was published under PCT Article 21(2) in English.

Field of the Invention

- 10 The present invention relates to anti-inflammatory peptides derived from pro-inflammatory IL-2, derivatives thereof, and to pharmaceutical compositions comprising said anti-inflammatory peptides.

Background of the Invention

- 15 The migration of T cells through tissues is regulated by adhesion receptors, such as integrins, and by receptors that receive signals provided by pro-inflammatory mediators, such as cytokines, chemokines, and extracellular matrix (ECM)-degrading enzymes (Gilat et al., 1996; Butcher and Picker, 1996).

- 20 IL-2 is a 15.5 kDa glycoprotein that participates in the development of inflammation and in the regulation of apoptosis (Taniguchi and Minami, 1993). In addition to its pro-activatory and proliferative roles, IL-2 also induces neutrophil adhesion to umbilical vein endothelial cells in a CD18-mediated manner (Li et al., 1996) as well as chemotactic responses in T cells, both directly and via regulating their expression of CC chemokine receptors (Loetscher et al., 1996). The IL-2
25 receptor consists of three distinct membrane chains: the α -, β - and γ -chains. Anti-IL-2 antibodies that recognize amino acid epitopes in the N-terminal region of IL-2 can inhibit IL-2-induced lymphocyte proliferation. The C-terminal portion of IL-2 and its three Cys residues seem to contribute to the folding and active conformation of IL-2 (Kuo and Robb, 1986).

- 30 The majority of neutrophil elastase (also termed human leukocyte elastase; HLE), which exists as either a membrane-bound or soluble moiety, is produced and released by neutrophils, although small amounts are also produced by macrophages, monocytes, and T cells (Packard et al., 1995).

Elastase degrades basement membrane and ECM glycoproteins, such as elastin, collagen, and fibronectin (FN), as well as molecules expressed on the surface of T cells, e.g., CD4, CD8, and CD2 (Doring et al., 1995).

5 **Summary of the Invention**

It has now been surprisingly observed, according to the present invention, that enzymatic degradation of IL-2 by proteolytic enzymes that participate in the breakdown of the extracellular matrix (ECM) results in small peptidic fragments that possess anti-inflammatory activity.

- 10 A first aspect of the present invention therefore relates to anti-inflammatory peptides derived from IL-2, and to anti-inflammatory derivatives of said peptides.

The anti-inflammatory peptides of the present invention are defined as such by virtue of their ability to inhibit at least one of the following processes in vitro: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-
15 induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells.

- 20 A second aspect of the present invention relates to a method for identifying anti-inflammatory peptides derived from IL-2, which comprises:

(i) carrying out enzymatic digestion of IL-2 with a proteolytic enzyme that participates in the breakdown of the extracellular matrix (ECM);

- (ii) testing the fractions obtained in (i) for their in vitro ability to inhibit at least one of the
25 following processes: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells;

- (iii) selecting the fractions of (ii) active in at least one of the bioassays (a) to (e),
30 fractionating each fraction to isolate individual peptides thereof, and submitting each isolated peptide to sequencing and synthesis; and

(iv) carrying out one or more of the bioassays (a) to (e) with a peptide isolated in step (iii), and selecting those peptides that exhibit anti-inflammatory activity in at least one of said bioassays.

- 5 The second aspect of the invention further relates to the peptides obtainable from the method of identification described above and peptide derivatives thereof.

10 The proteolytic enzyme is one capable of processing the glycoprotein constituents of tissues and blood vessel walls and may be elastase, a collagenase or a metalloprotease such as metalloelastase, matrix metalloprotease-2 (MMP-2) and matrix metalloprotease-9 (MMP-9). In a preferred embodiment, the proteolytic enzyme is elastase.

15 The anti-inflammatory synthetic peptides of the present invention have been characterized by their possession of inhibitory activity in at least one of processes (a) to (e) given above, the anti-inflammatory peptide being preferably active in at least one of assays (a), (b) or (e).

20 A third aspect of the invention relates to pharmaceutical compositions comprising at least one anti-inflammatory synthetic peptide of the invention and a pharmaceutically acceptable carrier. This aspect includes also the use of said peptides for the preparation of pharmaceutical compositions for the treatment and/or alleviation of acute and chronic inflammatory disorders.

A fourth aspect of the invention relates to a method for the treatment and/or alleviation of acute and chronic inflammatory disorders comprising administering to a subject in need thereof an effective amount of an anti-inflammatory synthetic peptide of the invention.

25

Brief Description of the Drawings

30 Figs. 1A-1B show induction of T cell adhesion to fibronectin (FN), laminin (LN), collagen type IV (CO-IV) (Fig. 1A) and ECM (Fig. 1B) by IL-2. [⁵¹Cr]-Labeled human T cells were seeded onto FN-, LN-, CO-IV-, and ECM-coated microtiter wells together with IL-2. After 30 min at 37°C, non-adherent T cells were removed, the adherent cells were lysed, and the percentage of T cells that had adhered was determined. One experiment representative of four.

Fig. 2 depicts a chromatogram of IL-2 fractions obtained upon elastase proteolysis. IL-2 (1mg/ml) was incubated (12 hr, 37°C) with neutrophil elastase (50µg/ml in PBS). The resulting enzymatic digests were purified by HPLC. Fraction 1 consisted of salts used for the separation procedure. One experiment representative of three.

Fig. 3 shows effects of IL-2 fractions, generated by elastase degradation, on the IL-2-induced adhesion of T cells to FN. IL-2 (10 U/ml)-stimulated [⁵¹Cr]-labeled T cells were seeded, in the presence or absence of elastase-generated IL-2 protein products, onto wells coated with FN. After 30 min at 37°C, non-adherent T cells were removed, and the percentage of adhered cells was determined. One experiment representative of six.

Figs. 4A-4C show specific inhibition, by elastase-generated fractions 2 and 8 of IL-2 and by their synthetic peptides, of IL-2-induced T cell adhesion to FN. Fig. 4A: T cell adhesion to FN in the presence of IL-2 fractions and the IL-2 peptides Ile-Val-Leu (IVL), Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (EFLNRWIT) (SEQ ID NO:1), and Arg-Met-Leu-Thr (RMLT) (SEQ ID NO:2), herein designated **pep1**, **pep2** and **pep3**, respectively. Fig. 4B. The effects on T cell adhesion to FN of the inversely synthesized IL-2 peptides Leu-Val-Ile (LVI), Thr-Leu-Met-Arg (TLMR) (SEQ ID NO:38) and Thr-Ile-Trp-Arg-Asn-Leu-Phe-Glu (TIWRNLFE) (SEQ ID NO:39). T cells were activated with IL-2 (10 U/ml) and seeded onto FN-coated wells in the presence of IL-2, fraction 2, fraction 8, or inversed IL-2 peptides. Fig. 4C. Effects of pre-treatments of T cells with the IL-2 peptides **pep1**, **pep2** and **pep3**, on the subsequent IL-2-induced T cell adhesion to FN. T cells used were untreated or pre-treated with the indicated peptides (1 pg/ml; 60 min, 37°C, 10% CO₂, humidified atmosphere), washed twice, exposed to IL-2, and seeded onto the FN-coated wells. After 30 min at 37°C, T cell adhesion was measured. One experiment representative of five.

Fig. 5 shows inhibition by the IL-2 peptides **pep1**, **pep2** and **pep3**, of T cell adhesion to LN, CO-IV and FN. T cells were pre-treated with the indicated IL-2 peptides (10 pg/ml, 30 min, 37°C, 10% CO₂, humidified atmosphere) and then with IL-2 (10 U/ml). The T cells were then

seeded onto microtiter wells that were pre-coated (1 μ g/well) with the various ECM glycoproteins. T cell adhesion was measured 30 min later.

Fig. 6 shows inhibition of IL-2-induced T cell migration through FN by **pep1** and **pep3**. T cells were pre-treated with the IL-2 peptides (1 pg/ml) or buffer alone and then placed in the upper wells of a chemotaxis chamber, in which IL-2 (10 U/ml) or MIP-1 β (10 ng/ml) had been added to the lower compartment. T cell migration towards the chemotactic sources was assessed after 2 hr. One experiment representative of four.

Figs. 7A-7B show inhibition by **pep2** (Fig. 7A) and **pep1** (Fig. 7B) of T cell adhesion to FN, induced by various activators. Labeled T cells were seeded onto FN-coated wells in the presence of IL-2 (10 U/ml), IL-7 (50 ng/ml), MIP-1 β (20 ng/ml), PMA (50 ng/ml), 8A2 mAb (1 μ g/ml), or anti-human CD3 mAb (1 μ g/ml). The IL-2-derived peptides, **pep1** or **pep2**, were also present in some wells. After 30 min at 37°C, non-adherent T cells were removed by washing, the remaining adherent cells were lysed, and the percentage of T cells that had adhered was determined. One experiment representative of four.

Figs. 8A-8E show inhibition by **pep2** of the spreading and redistribution of the actin cytoskeleton in IL-2- and PMA-activated FN-adherent T cells. T cells were activated (48 hr) with IL-2 (50 U/ml). The T cells were then washed and seeded onto FN-coated coverslips, in medium alone (8E), or in the presence of IL-2 (100 U/ml; 8A and 8C), PMA (50 ng/ml; 8B and 8D), or **pep2** (10 pg/ml; 8C and 8D). After incubation, the intracellular actin filaments of the fixed FN-attached T cells were stained. Original magnification, $\times 1000$.

Detailed Description of the Invention

IL-2 and elastase are prominent mediators of leukocyte extravasation and migration from the vasculature through the ECM to sites of inflammation. The present invention is based on the assumption that, in contrast to the pro-adhesive effects of the intact IL-2 molecule, certain short IL-2-derived peptides, which may occur in vivo, can inhibit the interactions of T cells with ECM, and that this interference is independent of the effects of the peptides on the activation of

T cells by IL-2. We also assumed that such moieties of IL-2 can prevent the arrival of T cells at inflamed sites.

An important feature of elastase is its ability to act in both soluble and immobilized forms, since a migrating immune cell that expresses immobilized elastase may encounter matrix-bound IL-2, among other cytokines. This made neutrophil elastase a likely candidate for the physiologic production of these inhibitory peptides. Indeed, it was found, according to the present invention, that the processing of recombinant IL-2 by elastase resulted in the production of at least eight different by-products.

Three of the products generated by processing of recombinant IL-2 by neutrophil elastase (present in HPLC fractions 2, 7 and 8), inhibited IL-2-mediated T cell adhesion to FN. Amino acid composition analysis and amino acid sequencing revealed that fraction 2 contained the tripeptide Ile-Val-Leu (IL-2₁₁₂₋₁₁₄) (herein designated **pep1**) and the tetrapeptide Arg-Met-Leu-Thr (IL-2₅₈₋₆₁) (SEQ ID NO:2) (herein designated **pep3**), and fraction 8 the octapeptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (IL-2₁₃₆₋₁₄₃) (SEQ ID NO:1) (herein designated **pep2**). The **pep3** appeared to be located within the IL-2-binding site of the α -chain of the IL-2R, whereas **pep1** and **pep2** are located at sites far from the receptor-binding sites of IL-2. These peptides, at a picomolar range of concentrations (i.e., 0.01-1 pg/ml), inhibited the IL-2, as well as MIP-1 β -induced chemotaxis of human T cells through FN-coated polycarbonate membranes.

These peptides were synthesized and the synthetic peptides were shown to exhibit the same activity as the IL-2 digested fractions 2 and 8. Thus, in one preferred embodiment, the IL-2 derived peptides of the invention are the peptides:

- (**pep1**) Ile-Val-Leu,
- (**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)
- (**pep3**) Arg-Met-Leu-Thr (SEQ ID NO:2),

The chemoattractive capacity of IL-2 in T cell migration studies in vitro has been shown by using bare polycarbonate filters or collagen- or Matrigel-coated membranes as immobilized

substrates. T cell migration in these systems was proved to be IL-2R β -chain-specific and depended on the activities of the matrix-degrading gelatinases (Leppert et al., 1995). According to the present invention, the IL-2 peptides **pep1**, **pep2**, **pep3**, in addition to their anti-migratory effects, also inhibited T cell adhesion to FN induced by various physiologic and non-physiologic stimuli. Nevertheless, none of them, at 1 to 100 pg/ml, interfered with either PHA- or IL-2-mediated proliferative responses of human T cells, nor did these peptides inhibit the secretion of TNF- α and IFN- γ from these proliferating cells (not shown). Moreover, the inversely synthesized molecules, Leu-Val-Ile, Thr-Leu-Met-Arg (SEQ ID NO:38), and Thr-Ile-Trp-Arg-Asn-Leu-Phe-Glu (SEQ ID NO:39), did not inhibit T cell adhesion to FN. Thus, the migration- and adhesion-suppressive capabilities of **pep1**, **pep2** and **pep3** are specific, and are not due to toxic cell death.

How do the elastase-derived IL-2 peptides exert their inhibitory functions? We have demonstrated, according to the present invention, that the three peptides **pep1**, **pep2** and **pep3** do not have to be present during the entire period of the adhesion assay, since their anti-adhesive effect was apparent even after their removal from the assay prior to T cell activation with IL-2. This finding also implies that **pep1**, **pep2** and **pep3** do not function by interacting with putative cell-adhesive epitopes present on the tested ECM glycoproteins. It seems to be highly unlikely that two of these peptides (**pep1** and **pep2**) exert their biologic functions by interacting with the IL-2R subunits or by directly binding to β_1 -specific integrins. The IL-2 peptides interfered with different modes of T cell activation, leading to their adhesion to the tested matrix proteins. Moreover, both **pep1** and **pep2**, used in a picomolar range of concentrations, appear to block T cell adhesion not only to FN, mediated predominantly via the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, but also to LN; T cell adhesion to LN was mediated primarily through the $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins. Interesting, however, is the fact that **pep3** (Arg-Met-Leu-Thr (SEQ ID NO:2)) resides within the IL-2R α -binding site of IL-2; two residues, Arg⁵⁸ and Phe⁶², which are present within and adjacent to this peptide, respectively, were shown to be critical for IL-2-IL-2R interactions. Therefore, it will also be interesting to examine whether the degradation of IL-2 by elastase produces compounds that can interfere with IL-2 binding to its receptor and with the biologic outcome of such molecular interactions.

The ability of **pep2** as well as **pep1** and **pep3** (data not shown) to inhibit the PMA- and IL-2-induced T cell adhesion to FN is probably linked to its ability to block the reorganization of the intracellular actin cytoskeleton. Integrin-cytoskeleton associations can modulate cell adhesion to ECM ligands, cell spreading in areas of cell contact with the substratum, and the micro-clustering and redistribution of β_1 integrins on the cell surface at sites of focal adhesion located at the ends of the actin fibers (Otey et al., 1990). Similar observations were noted for T cell activation by IL-2 (and PMA) and attachment to FN. Although the intracellular mechanisms of action of the elastase-generated fractions and peptides have not yet been determined, we postulate that these proteins effect the adhesion and migration of T cells within the ECM by active inhibition of intracellular signal transduction pathways linked to cytoskeleton organization, resulting in an inhibition of micro-clustering and an association of integrins with cytoskeletal elements.

Our findings according to the present invention imply that the tissue-invading T cells themselves can dynamically regulate their own functions. Both adhesion- and migration-promoting stimuli (i.e. intact IL-2) and suppressive by-products of inflammatory mediators can be present, although not necessarily simultaneously, within the inflammatory milieu. At the early stages of inflammation, both IL-2 and elastase may function concomitantly to activate T cells to penetrate tissues. Later, the degradation peptide products of IL-2, generated by elastase, may inhibit T cell migration, inhibit the co-stimulatory effects of IL-2 and other mediators, and probably signal the termination of the inflammatory reaction.

As stated before, the invention relates to anti-inflammatory peptides derived from the IL-2 sequence and to derivatives thereof, said anti-inflammatory activity being assessed by their ability to inhibit at least one of the following processes in vitro: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells.

The in vitro bioassays (a) to (e) are well-known in the art. Thus, for example, assays (a), (b) and (e) may be carried out as described in the section Materials and Methods hereinafter. Assay (c), for inhibition of cytokine - or mitogen-induced T cell proliferation, is carried out as follows: A total of 25×10^5 of human T cells in 0.2 ml of RPMI medium supplemented with 5% heat-inactivated FCS (Gibco), antibiotics, 1 % Hepes buffer, 1% glutamine and 5×10^{-5} M 2-mercaptoethanol (ME), are added per well. T cell activation is tested by adding either the cytokine IL-2 (100 U/ml) or the mitogen Con-A (2.5 μ g/ml) to the culture wells in the beginning of the assay. The IL-2-derived peptides or their derivatives are added to the culture wells (different amounts, 10 microliters) for the entire period of the assay. [3 H]-Thymidine is added to the microtiter 96 U-form well plates 18h before harvesting the cultures on day 3 onto glass fiber filters and counted by liquid scintillation counter. Mean CPM and SD from quadruplicate culture are then presented. Assay (d), for inhibition of cytokine secretion by cytokine- or mitogen-stimulated T cells, is carried out similar to the proliferation assay (c), except for one modification: the supernatants of activated T cells (or any other leukocytes derived from human blood), treated with the different peptides, are collected and assayed for IL-2 and TNF- α , using conventional ELISA kits.

In vivo bioassays may also be used to determine anti-inflammatory activity of the peptides of the invention, examples of which include: (1) inhibition of experimental delayed type hypersensitivity (DTH) reactivity carried out, for example, as described in PCT Publication No. WO 94/11006, Example 5.2; (2) treatment of experimental adjuvant arthritis (AA) in rats carried out, for example, as described in PCT Publication No. WO 94/11006, Example 5.7; and (3) treatment of experimental autoimmune encephalomyelitis (EAE) in guinea pigs, a model disease for multiple sclerosis, carried out, for example, as described in US Patent No. 5,206,223.

The term "peptides of the invention" as used hereinafter includes the synthetic peptides **pep1**, **pep2**, and **pep3** derived from IL-2 as well as synthetic analogues thereof, and comprise:

(i) peptides **pep1**, **pep2**, and **pep3** of the sequences:

(**pep1**) Ile-Val-Leu

(**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)

(**pep3**) Arg-Met-Leu-Thr (SEQ ID NO:2)

(ii) peptides obtained from **pep2** by deletion of one or more amino acid residues;

(iii) peptides obtained by addition to peptides (i) or (ii) of one or more natural or non-natural amino acid residues;

(iv) peptides obtained by replacement of one or more amino acid residues of peptides (i) to (iii) by the corresponding D-stereomer, by another natural amino acid residue or by a non-natural amino acid residue;

(v) chemical derivatives of the peptides (i) to (iv);

(vi) cyclic derivatives of peptides (i) to (v);

(vii) dual peptides consisting of two of the same or different peptides (i) to (vi), wherein the peptides are covalently linked to one another directly or through a spacer; and

(viii) multimers comprising a number of the same or different peptides (i) to (vi).

The term “peptide derivative” or “peptide analogue” as used throughout the specification and claims herein is intended to include the derivatives defined in (ii) to (viii) above, namely peptides obtained by deletion, addition, or replacement of a natural amino acid residue by the corresponding D-stereomer or by a non-natural amino acid residue, chemical derivatives of the peptides, cyclic peptides, dual peptides and multimers of the peptides.

Typically, modifications are made that retain the anti-inflammatory effect of the parent peptides **pep1**, **pep2** and **pep3**. Any of the above modifications may be utilized alone or in combination, provided that the modified sequence retains anti-inflammatory activity, preferably at least 30% of the anti-inflammatory activity, more preferably at least the same activity or higher, of the parent peptide, identified by means of the above described assays.

Deletion of 1-4 amino acid residues of **pep2** may be made at the N- or the C-terminal such as for example peptides **pep19** and **pep21**, respectively, in Table 1 hereinafter. Addition of one or more natural or non-natural amino acid residues may be made at the N- or the C-terminal of each of the parent peptides **pep1**, **pep2** and **pep3**, such as for example peptides **pep4**, **pep5** and **pep6** (analogues of **pep1**), the peptides **pep15** and **pep17** (analogues of **pep2**), and the peptide **pep43** (analogue of **pep3**).

Substitutions include replacement of the natural amino acid residues by the corresponding D-amino acid residue, for example to increase blood plasma half-life of a therapeutically administered peptide, or by different natural amino acid residues or by non-natural amino acid residues. Thus, the peptide or peptide derivative of the invention may be all-L, all-D or a D,L-peptide. Examples of non-natural amino acids include, but are not limited to, N α -alkyl, particularly N α -methyl, amino acids*, C α -alkyl, particularly C α -methyl, amino acids*, halo derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine* and p-I-phenylalanine*, β -alanine (β -Ala)*, L- α -aminobutyric acid*, L- γ -aminobutyric acid (γ -Abu)*, L- α -aminoisobutyric acid (α -Aib)*, L- ϵ -aminocaproic acid*, 7-aminoheptanoic acid*, L-norleucine (Nle)*, L-norvaline (Nva)*, L-p-nitro-phenylalanine*, L-hydroxyproline (Hyp)*, L-thioprolin*, ornithine (Orn)^{#*}, homoarginine (homoArg)[#], diaminobutyric acid (Dab)^{#*}, pyridylalanine*, thienylalanine*, naphthylalanine*, phenylglycine*, methyl derivatives of phenylalanine (Phe) such as L-4-methyl-Phe* and L-pentamethyl-Phe*, L-4-amino-Phe*, L-4-isopropyl-Phe*, L-4-phenyl-Phe*, L-4-benzyl-Phe*, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (L-Tic)*, L-methyl-Tyr*, and L-diaminopropionic acid (Dap)^{#*}. The notation * has been utilised to indicate the hydrophobic nature of the moiety whereas # has been utilised to indicate the hydrophilic nature of the derivative, and #* indicates amphipathic characteristics.

A "chemical derivative" of a peptide of the invention includes, but is not limited to, a derivative containing additional chemical moieties not normally a part of the peptide provided that the derivative retains the anti-inflammatory function of the peptide. Examples of such derivatives are: (a) N-acyl derivatives of the amino terminal or of another free amino group, wherein the acyl group may be either an alkanoyl group, e.g. acetyl, hexanoyl, octanoyl, or an aroyl group, e.g. benzoyl; (b) esters of the carboxy terminal or of another free carboxy or hydroxy groups; (c) amides of the carboxy terminal or of another free carboxy groups produced by reaction with ammonia or with a suitable amine, resulting in the C-terminus or another carboxy group being in the form -C(O)-NH-R, wherein R may be hydrogen, C1-6 alkyl, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl or hexyl, aryl such as phenyl, and aralkyl such as benzyl, such amidation being advantageous in providing additional stability and possibly enhanced activity to

the peptide; (d) glycosylated derivatives; (e) phosphorylated derivatives; (f) derivatives conjugated to lipophilic moieties e.g. caproyl, lauryl, stearoyl; and (g) derivatives conjugated to an antibody or other cellular ligands. Also included among the chemical derivatives are those derivatives obtained by modification of the peptide bond -CO-NH-, for example by (a) reduction to -CH₂-NH-; (b) alkylation to -CO-N (alkyl)-; (c) inversion to -NH-CO-.

The term "cyclic peptides" as used herein are cyclic derivatives containing either an intramolecular disulfide bond, i.e. -S-S-, an intramolecular amide bond, i.e. -CONH- or -NHCO-, or intramolecular S-alkyl bonds, i.e. -S-(CH₂)_n-CONH- or -NH-CO(CH₂)_n-S-, wherein n is 1 or 2. The cyclic derivatives containing an intramolecular disulfide bond may be prepared by conventional solid phase synthesis while incorporating suitable S-protected cysteine or homocysteine residues at the positions selected for cyclization such as the amino and carboxy terminals of the peptides, with the option of including spacing residues, such as (Ala)_n, (Gly)_n where n is from 1 to 4, or non-natural amino acids such as 6-aminocaproic acid, between the terminal residue and the linking residue. The linking residues may then be linked together using known techniques to form cyclicized peptide derivatives. For example, the linear peptides Cys-Ile-Val-Leu-Ala-Cys (SEQ ID NO:6), Cys-Ile-Val-Leu-Ala-Ala-Cys (SEQ ID NO:7), Cys-Arg-Met-Leu-Thr-Ala-Cys (SEQ ID NO:37) and Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys (SEQ ID NO:27) were prepared and cyclized according to methods known in the art for formation of a disulphide bond. Following completion of the chain assembly, cyclization can be performed either by selective removal of the S-protecting groups with a consequent on-support oxidation of free corresponding two SH-functions, to form S-S bonds, followed by conventional removal of the product from the support and appropriate purification procedure, or by removal of the peptide from the support along with complete side-chain deprotection, followed by oxidation of the free SH-functions in highly dilute aqueous solution. The cyclic derivatives containing an intramolecular amide bond may be prepared by conventional solid phase synthesis while incorporating suitable amino and carboxyl side-chain protected amino acid derivatives at the positions selected for cyclization. The cyclic derivatives containing intramolecular -S-alkyl bonds may be prepared by conventional solid phase synthesis while incorporating an amino acid residue with a suitable amino-protected side chain, and a suitable S-protected cysteine or homocysteine residue at the positions selected for cyclization.

A "dual peptide" according to the invention consists of two the same or different peptides or peptide derivatives of the invention covalently linked to one another directly or through a spacer such as by a short stretch of alanine residues or by a putative site for proteolysis by cathepsin (see U.S. Patent No. 5,126,249 and European Patent No. 495,049 with respect to such sites). This will induce site-specific proteolysis of the preferred form into the two desired analogues.

"Multimers" according to the invention consist of polymer molecules formed from a number of the same or different peptides or derivatives thereof. The polymerization is carried out with a suitable polymerization agent, such as 0.1% glutaraldehyde (Audibert et al. (1981) Nature, 289 : 593)

Modifications to the amino acid residues in the peptides of the present invention are discussed below using nomenclature as given in the Table below:

ALIPHATIC	Non-polar, neutral	Gly ₂ Ala ₂ Pro
Low hydrophobic		
High hydrophobic		Ile ₂ Leu ₂ Val ₂ Met
Low hydrophilic	Polar, neutral	Cys ₂ Ser ₂ Thr
High hydrophilic		Asn ₂ Gln
Hydrophilic	Polar – negatively charged	Asp ₂ Glu
	Polar – positively charged	Lys ₂ Arg ₂ His
AROMATIC hydrophobic		Phe ₂ Trp ₂ Tyr

Thus, in one preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Ile-Val-Leu (**pep1**), and to anti-inflammatory peptides resulting from the modification thereof by:

(a) elongation by up to 3-4 further amino acid residues at the N- and/or C-terminal, such as in **pep11** and **pep13**, or preferably according to the natural sequence of IL-2;

(b) substitution of the Ile residue by a natural or non-natural amino acid hydrophilic polar neutral or negatively charged, or hydrophobic non-polar neutral amino acid residue, preferably selected from Glu, Asp, Asn, Gln, Ala, Val;

(c) substitution of the Val residue by a hydrophobic, non-charged natural or non-natural amino acid residue, preferably selected from Ala, Ile, Leu, Met, Nle, Phe;

(d) substitution of the Leu residue by a hydrophobic, non-charged natural or non-natural amino acid residue, preferably selected from Ala, Ile, Met, Nle, Phe, Val;

(e) amidation of the C-terminal Leu residue,

(f) cyclization of **pep1** or of any peptide of (a) to (e); and

(g) any combination of (a) to (f).

Examples of such modified peptides derived from **pep1** include;

(**pep4**) Asn-Ile-Asn-Val-Ile-Val-Leu (SEQ ID NO:3),

(**pep5**) Ile-Val-Leu-Glu-Leu-Lys-Gly (SEQ ID NO:4),

(**pep6**) Asn-Val-Ile-Val-Leu (SEQ ID NO:5)

(**pep7**) Ala-Val-Leu

(**pep8**) Ile-Ala-Leu

(**pep9**) Ile-Val-Ala

(**pep10**) Glu-Val-Leu

(**pep11**, linear) and (**pep12**, cyclic) Cys-Ile-Val-Leu-Ala-Cys (SEQ ID NO:6) and,

(**pep13**, linear) and (**pep14**, cyclic) Cys-Ile-Val-Leu-Ala-Ala-Cys (SEQ ID NO:7).

In a further preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (**pep2**) (SEQ ID NO:1), and to anti-inflammatory peptides resulting from the modification thereof by;

(a) elongation by up to 4 further amino acid residues at the C and/or N terminal ends, such as in **pep34**, or preferably according to the natural sequence of IL-2;

(b) substitution of the Glu residue by a natural or non-natural charged or polar charged amino acid residue, preferably selected from Lys, Arg, Asp, Gln, Asn;

(c) substitution of the Phe residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(d) substitution of the Leu residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(e) substitution of the important Asn residue by a hydrophilic, non-charged, aliphatic natural or non-natural amino acid residue such as Gln;

(f) substitution of the Arg residue by a positively charged, natural or non-natural amino acid residue, preferably selected from Lys, Orn, homoArg;

(g) substitution of the Trp residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Ile, Leu, Nle, Tic, Phe, 4-phenyl-Phe, 4-methyl-Phe;

(h) substitution of the Ile residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Phe, Leu, Nle, Tic;

(i) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue preferably selected from Cys, Ser;

(j) truncation by up to 4 amino acid residues from either the C or N terminal;

(k) amidation of the C-terminal Thr;

(l) cyclization of **pep2** or of any peptide of (a) to (k); and

(m) any combination of (a) to (l).

Examples of such modified peptides derived from **pep2** include:

- | | |
|------------------|---|
| (pep15) | Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (<u>SEQ ID NO:8</u>) |
| (pep16) | Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys (<u>SEQ ID NO:9</u>) |
| (pep17) | Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (<u>SEQ ID NO:10</u>) |
| (pep18) | Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser (<u>SEQ ID NO:11</u>) |
| (pep19) | Leu-Asn-Arg-Trp-Ile-Thr (<u>SEQ ID NO:12</u>) |
| (pep20) | Arg-Trp-Ile-Thr (<u>SEQ ID NO:13</u>) |
| (pep21) | Glu-Phe-Leu-Asn (<u>SEQ ID NO:14</u>) |

(pep22) Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:15)
 (pep23) Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:16)
 (pep24) Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:17)
 ((pep25) Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:18)
 5 (pep26) Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:19)
 (pep27) Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr (SEQ ID NO:20)
 (pep28) Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr (SEQ ID NO:21)
 (pep29) Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr (SEQ ID NO:22)
 (pep30) Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr (SEQ ID NO:23)
 10 (pep31) Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr (SEQ ID NO:24)
 (pep32) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala (SEQ ID NO:25)
 (pep33) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-NH₂ (SEQ ID NO:26) and,
 (pep34, linear) and (pep35, cyclic) Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys
 (SEQ ID NO:27).

In a further preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Arg-Met-Leu-Thr (**pep3**), and to anti-inflammatory peptides resulting from the modification thereof by:

- (a) elongation by up to 4 further amino acid residues at the C and/or N terminal end, such as in **pep45**, or preferably according to the natural sequence of IL-2;
- (b) substitution of the Arg residue by a natural or non-natural positively charged amino acid residue, preferably selected from Lys, Orn, homoArg, diaminobutyric acid;
- (c) substitution of the Met residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Ile, Leu, Nle, Tic;
- (d) substitution of the Leu residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Nle, Tic;
- (e) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue such as Ser, Cys;
- (f) amidation of the C-terminal Thr residue;
- (g) cyclization of **pep3** or of any peptide of (a) to (f); and
- (h) any combination of (a) to (g).

Examples of such modified peptides derived from **pep3** include;

- (**pep36**) Ala-Met-Leu-Thr (SEQ ID NO:28)
(**pep37**) Arg-Ala-Leu-Thr (SEQ ID NO:29)
5 (**pep38**) Arg-Met-Ala-Thr (SEQ ID NO:30)
(**pep39**) Arg-Met-Leu-Ala (SEQ ID NO:31)
(**pep40**) Lys-Met-Leu-Thr (SEQ ID NO:32)
(**pep41**) Arg-Val-Leu-Thr (SEQ ID NO:33)
(**pep42**) Arg-Met-Leu-Thr-NH₂ (SEQ ID NO:34)
10 (**pep43**) Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr (SEQ ID NO:35)
(**pep44**) Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr (SEQ ID NO:36) and,
(**pep45**, linear) and (**pep46**, cyclic) Cys-Arg-Met-Leu-Thr-Ala-Cys (SEQ ID NO:37).

The peptides and peptide derivatives of the invention are obtained by any method of peptide
15 synthesis known to those skilled in the art, such as for example by solid phase peptide synthesis.

The present invention is also directed to pharmaceutical compositions comprising a
pharmaceutically acceptable carrier and at least one anti-inflammatory peptide or peptide
derivative of the invention. The pharmaceutical composition will be administered according to
20 known modes of peptide administration, including oral, intravenous, subcutaneous,
intraarticular, intramuscular, inhalation, intranasal, intrathecal, intradermal, transdermal or other
known routes. The dosage administered will be dependent upon the age, sex, health condition
and weight of the recipient, and the nature of the effect desired.

25 The peptides of the invention for use in therapy are typically formulated for administration to
patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical
composition. The formulation will depend upon the nature of the peptide and the route of
administration but typically they can be formulated for topical, parenteral, intramuscular,
intravenous, intraperitoneal, intranasal inhalation, lung inhalation, intradermal or intra-articular
30 administration. The peptide may be used in an injectable form. It may therefore be mixed with any

vehicle which is pharmaceutically acceptable for an injectable formulation, preferably for a direct injection at the site to be treated, although it may be administered systemically.

The pharmaceutically acceptable carrier or diluent may be, for example, sterile isotonic saline solutions, or other isotonic solutions such as phosphate-buffered saline. The peptides of the present invention may be admixed with any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s). It is also preferred to formulate the peptide in an orally active form.

Tablets or capsules of the peptides may be administered singly or two or more at a time, as appropriate. It is also possible to administer the peptides in sustained release formulations.

Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient.

There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Alternatively, the peptides of the invention, can be administered by inhalation or in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. An alternative means of transdermal administration is by use of a skin patch. For example, they can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. They can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.

For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents. For such oral administration, the peptide may preferably be formed into microcapsules or nanoparticles together with biocompatible polymers such as poly-lactic acid and the like.

The compositions (as well as the peptides alone) can also be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. In this case, the compositions will comprise a suitable carrier or diluent. For parenteral administration, the compositions are best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood.

For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The peptides and peptide derivatives of the invention are for use in the treatment of or amelioration of acute and chronic inflammatory disorders including, but not being limited to, autoimmune diseases such as rheumatoid arthritis, diabetes type I, multiple sclerosis, systemic lupus erythematosus, uveitis, bowel inflammation and Crohn's disease.

The present invention further relates to a method of treatment of a patient suffering from an inflammatory disorder which comprises administering to said patient an effective amount of a peptide or peptide derivative of the invention.

The invention will now be illustrated by the following non-limiting Examples.

EXAMPLES

Material and methods

Materials. The following reagents were obtained as indicated. Recombinant human IL-2 (sp. act. 18×10^6 U/mg; Chiron B.V., Amsterdam, The Netherlands); recombinant human IL-7 (sp. act. 2×10^5 U/mg; Immunex Corp., Seattle, WA); recombinant human macrophage inflammatory protein MIP-1 β (Pepro Tech, Rocky Hill, NJ); recombinant human TNF- α (Boehringer, Ingelheim, Germany); Affi-gel 10 beads (Pharmacia, NJ); fibronectin (FN) (Chemicon; Temecula, CA); BSA, laminin (LN), PMA, and TRITC-conjugated phalloidin

(Sigma Chemical Co., St. Louis, MO); collagen type IV (CO-IV; ICN, Costa Mesa, CA); and HEPES buffer, antibiotics, heat-inactivated FCS, sodium pyruvate, DMEM, CSS and RPMI-1640 (Beit-Haemek, Israel). An anti- β_1 integrin-specific affinity-modulating mAb, 8A2, was donated by Dr. J. M. Harlan (Washington University, Seattle, WA). All protected amino acids, coupling reagents, and polymers were obtained from Nova Biochemicals (Läufelfingen, Switzerland). Synthesis-grade solvents were obtained from Labscan (Dublin, Ireland). HPLC solvents and columns were obtained from Merck (Darmstadt, Germany).

Human colonic epithelial cell lines HT-29 (ATCC HTB38) and Caco-2 (ATCC HTB27) were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in culture medium (DMEM supplemented with 10% cosmic calf serum (CSS), 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin), at 37°C and atmosphere of 5% CO₂. Cell viability was assessed using the standard MTT method.

T cell adhesion assays. Human T cells were purified from the peripheral blood of healthy donors, and T cell adhesion to immobilized protein substrates was examined as follows: Human leukocytes were isolated on a Ficoll gradient, washed, and incubated (2 h, 37°C, 7.5% CO₂, humidified atmosphere) on petri dishes. The non-adherent cells were then collected and incubated (1 h, 37°C, 7.5% CO₂, humidified atmosphere) on nylon wool columns (Novamed Ltd., Jerusalem, Israel). Unbound cells were eluted from the columns by extensive washings. The resulting cell population was always >92% T cells. Intact IL-2 or IL-2-derived peptides (in 50 μ l) were added to flat-bottom microtiter wells that had been pre-coated with ECM or ECM proteins (FN or LN; 1 μ g/well) and blocked with 0.1% BSA. After 4-6 h at 37°C, the wells were washed and ⁵¹[Cr]-labeled T cells were added to the wells, 10⁵ cells/100 μ l of adhesion medium (RPMI-1640 supplemented with 0.1% BSA, 1% sodium pyruvate, 1% HEPES buffer). The microtiter plates containing the cells were incubated (30 min, 37°C) in a humidified, 7.5% CO₂ atmosphere, and then washed. The adherent cells were lysed, and the resulting supernatants were removed and analyzed in a γ -counter. For each experimental group, the results were expressed as the mean percentage \pm SD of bound T cells from quadruplicate wells. To some wells, different concentrations of soluble IL-2 were added concomitantly with the T cells, and

with others, different concentrations of elastase-degraded IL-2-derived fractions, or the corresponding synthetic peptides, were added together with stimulators [PMA (50 ng/ml), IL-7 (50 ng/ml), MIP-1 β (20 ng/ml), 8A2 (1 μ g/ml), mAb anti-CD3 (1 μ g/ml), or IL-2 (10 U/ml)].

5 *Chemotaxis assays.* T cell chemotaxis was performed and analyzed as previously described (Ben-Baruch et al., 1997). Briefly, the migration of human T cells (0.5×10^6 cells in adhesion medium/well) was examined in a 48-well chemotaxis micro-chamber (Neuro-Probe Inc., Cabin John, MD). The two compartments of the micro-chambers were separated by a FN-coated polycarbonate filter (5- μ m pore size; Osmonics Protein Products, Livemore, CA). Where
10 indicated, MIP-1 β or IL-2 was added to the lower wells, and the T cells were added to the upper chambers together with the peptides. After incubation (120 min, 37°C, in a humidified, 7.5% CO₂ atmosphere), the filters were removed, fixed, and stained with a Diff-Quik staining kit (Dade, Dürdingen, Switzerland). The number of migrating T cells in five high-power fields (under 500x magnification; WILD Microscope, Heerbrugg, Switzerland) was evaluated. For
15 each group, the results are expressed as the mean number of cells in one high-power field.

Spontaneous or cytokine-induced secretion of IL-8 or IL-1 β from intestinal epithelial cell. Test cells (HT-29 or Caco-2) were grown as confluent monolayers in 24-well tissue culture plates. After the cells reached confluence, the culture medium was changed and the cells (10^6
20 cells/well) were incubated for 24 h at 37°C with the IL-2 peptides (or control and derivative peptides) in 24-well plates without (spontaneous) or with the addition of TNF- α (TNF- α -induced). When TNF- α was used, the peptides were added to the cells 1 hour prior to the addition of TNF- α . Following culture, the supernatants were harvested and analyzed for IL-8 or IL-1 β secretion by ELISA using commercially available kits for IL-8 (Pharmingen) and IL-1 β
25 (Genzyme), according to the manufacturers' protocols.

Purification of elastase and elastase digestion of IL-2. Neutrophils were isolated from the whole blood of a healthy donor by dextran sedimentation and Ficoll-Hypaque gradient centrifugation, as previously described (Yavin et al., 1996). Elastase was isolated by aprotinin-
30 Sepharose affinity chromatography, followed by carboxymethyl-cellulose ion exchange

chromatography, as developed by Baugh and Travis (Yavin et al., 1996; Baugh and Travis, 1976). The purified elastase, which was lyophilized and stored at -20°C until used, was biochemically checked to be entirely free from cross-contamination with cathepsin G (not shown). IL-2 was dissolved in distilled water to yield a 1mg/ml solution. Lyophilized neutrophil elastase (50 µg) was dissolved in 1 ml of PBS and immediately added to the IL-2 solution. The elastase-IL-2 mixture was incubated (12 h) at 37°C. Aliquots were removed and stored at -20°C until subjected to HPLC separation.

Reverse phase HPLC. Elastase digests of IL-2 were purified with a prepacked Lichrospher-100 RP-18 column (4x25 mm, 5 µm bead size), using a binary gradient formed with 0.1% trifluoroacetic acid (TFA) in H₂O (solution A) and 0.1% TFA in 75% acetonitrile in H₂O [(solution B) at $t=0$ min, B=3.5%, at $t=5$ min, B=3.5%, and then, the concentrations began to increase: at $t=60$ min, B=100% (i.e. 75% acetonitrile)]. The flow-rate was constant on 0.8ml/min. A Spectra-Physics SP8800 liquid chromatography system (Fremont, CA) equipped with an Applied Biosystems 757 (Foster City, CA) variable wavelength absorbency detector was used. The column effluents were monitored by UV absorbency at 220 nm, and the chromatograms were recorded on a ChromeJet integrator. Fractions that were 20% or more above valley levels were pooled, rotoevaporated to a minimal volume, and diluted with HPLC grade water. The rotoevaporation and dilution with water step was performed twice to remove residual TFA and acetonitrile.

Amino acid composition of the synthetic peptides and amino acid sequence analysis. Purified peptide solutions [≈ 40 µg of peptide in 40 µl, with 5 µg of norvaline (an unnatural amino acid) as an internal standard] were rotoevaporated, hydrolyzed (10°C, 22 h) in 6N HCl under vacuum, and analyzed with an amino acid analyzer (HP1090, Palo Alto, CA). An on-line pre-column ortho-phthalaldehyde (OPA)/9-fluorenylmethoxycarbonyl (F-moc) derivatization, combined with reverse phase chromatography, was used to determine the amino acid composition of the peptides and the total peptide yield. Without exception, all of the peptides yielded excellent analysis ratios of corresponding amino acids deviations from expected values of less than 10%. Analysis of the elastase-generated IL-2 fractions was performed using a Model 470A gas phase microsequencer. Phenylthiohydantoin amino acid derivatives were

separated on-line by reverse phase HPLC on a PTH C-18 column (2.1 x 220 mm) using a Model 120A analyzer (Applied Biosystems, CA).

Solid phase peptide synthesis. IL-2-derived peptides were prepared by conventional solid phase peptide synthesis, using an AMS-422 automated solid phase multiple peptide synthesizer (Abimed, Langenfeld, Germany). The Fmoc strategy was used for peptide chain assembly, according to the commercial protocol. In each reaction vessel, we used 12.5 μ mol of Wang resin containing the first covalently bound corresponding N-Fmoc C-terminal amino acid (typically, polymer loadings of 0.3-0.7 mmol/g resin were used). Fmoc deprotection was achieved by two consecutive treatments with 20% piperidine in dimethyl formamide, usually 10-15 min each at 22°C, depending on the length of peptide and the Fmoc-protected amino acid type. The protecting groups used for the side chain of the amino acids were tert-butyloxycarbonyl for Trp and Lys; trityl for Asn, Cys, Gln and His; tert-butylester for Asp and Glu; and tert-butylether for Ser, Tyr and Thr. Usually, coupling was achieved using two successive reactions (typically 20-45 min each at 22°C, depending on the length of peptide and amino acid derivative type) with 50 μ mol (4 eqv) of N-Fmoc-protected amino acid, 50 μ mol (4 eqv) of benzotriazole-1-yl-oxy-tris-pyrrolidino-phosphonium hexafluorophosphate (PyBop) reagent, and 100 μ mol (8 eqv) of N-methylmorpholine were all dissolved in dimethylformamide (DMF). The peptide was cleaved from the polymer by reacting (2 h, 22°C) the resin with trifluoroacetic acid/H₂O/triethylsilane (90/5/5; v/v/v). The solution containing the crude, unprotected peptides was then cooled down to 4°C, precipitated with ether (4°C), and centrifuged (15 min, 3000 rpm, 4°C). The pellet was washed and centrifuged (x3) with ether, dissolved in 30% acetonitrile in H₂O, and lyophilized. The lyophilized material was reconstituted in double distilled water before use; only the stock solution, not the diluted material, was stored at -20°C.

Synthesis of cyclic peptides. The linear peptides **pep12**, **pep14**, **pep35**, **pep46** were cyclized as follows: 0.5-1.0 mg of SH-free peptide was dissolved in 1 ml of ammonium acetate buffer (0.1N, pH 7.0). A solution of K₃ [Fe(CN)₆] (1-5mM) in the same buffer was slowly added to the stirred peptide solution at room temperature, in a titrimetric fashion until a stable faint

yellowish color was apparent. The solution revealed a negative 5,5'-dithio-bis(2-nitrobenzoic acid (DTNB; Ellman's Reagent)-test for free SH. Lyophilization and purification by HPLC were then performed as usual.

- 5 *Staining of actin cytoskeleton.* T cells were incubated (18 h, 37°C, 7.5% CO₂, humidified atmosphere) in culture medium. IL-2 or PMA was added to the cell cultures, which were then incubated for 24 h. The T cells were then washed and seeded onto FN-covered coverslips in the presence of either PMA (50 ng/ml), IL-2 (100 U/ml), or IL-2 peptides (0.1 ng/ml). After 1 h at 37°C, the adherent cells were fixed (3 min) with paraformaldehyde (3%) and Triton X-100 (0.5%), washed, and fixed (20 min) again with paraformaldehyde (3%). The fixed adherent cells were washed, treated with TRITC-phalloidin, and washed again. Photographs (x1000 magnification) were then taken.

Example 1

- 15 *Induction of the adhesion of T cells to ECM, FN, LN, and CO-IV by IL-2.* We examined the ability of soluble IL-2 to induce adhesion of human T cells to ECM, FN, LN, and CO-IV. The results indicated that IL-2 induced T cell adhesion to FN, LN, and CO-IV (Fig. 1A), as well as to intact ECM (Fig. 1B). Note that the adhesion of T cells to LN induced by IL-2 was lower than that induced to the other ECM glycoproteins. When T cells were activated only with PMA, 20 45±4.4% of them adhered to immobilized ECM and ECM glycoproteins (not shown). IL-2-induced T cell adhesion to the ECM glycoproteins was inhibited by anti-human β_1 integrin mAbs (not shown), which suggests that the pro-adhesive effects of IL-2 were induced via cell surface-expressed integrins. However, under our experimental conditions, IL-2 did not alter the T cell surface expression of β_1 integrins (not shown). Thus IL-2, in addition to other pro- 25 inflammatory mediators, appears to regulate the adhesiveness of resting human T cells to immobilized ECM and ECM glycoproteins.

Example 2

- 30 *The effects of IL-2 fragments obtained by elastase degradation on the interaction of T cells with FN.* We have assumed that the degradation of IL-2 can occur in the inflamed milieu in

which both cytokines, such as IL-2, and proteolytic enzymes, such as neutrophil elastase, are present. We also hypothesized that, in contrast to the intact IL-2 molecule, certain portions of IL-2 can abrogate the adhesiveness of activated T cells to ECM ligands. Hence, elastase and soluble IL-2 were incubated together at physiologic conditions. HPLC analysis of the elastase-degraded IL-2 revealed at least 8 peaks of IL-2, each of which represented at least one low molecular weight protein fragment (Fig. 2).

Next, we examined the ability of the HPLC-purified IL-2 fractions, which were generated by elastase-degradation, to inhibit soluble IL-2-induced interactions of T cells with FN. We chose to investigate the major peaks of HPLC-purified, elastase-degraded fractions of IL-2. Fractions 2, 7, and 8 inhibited the adhesion of T cells to immobilized FN in a dose-dependent and statistically significant fashion, whereas fractions 4 and the HPLC buffer did not (Fig. 3). Thus, certain IL-2 fragments, obtained by neutrophil elastase-processing of the cytokine, can inhibit IL-2-induced adhesion of T cells to FN.

Example 3

The effect of synthetic peptides, with putative amino acid compositions corresponding to fractions 2 and 8, on IL-2-induced T cell adhesion to FN. Next, the primary sequence of fractions 2 and 8 were analyzed by gas phase chromatography, because these elastase-generated fractions of IL-2 appeared to contain adhesion-suppressive peptides. Our analysis revealed that fragment 2 contained an Ile-Val-Leu (IVL; **pep1**; IL-2₁₁₂₋₁₁₄) and an Arg-Met-Leu-Thr (RMLT; **pep2**; IL-2₅₈₋₆₁) peptide (SEQ ID NO:2), whereas fragment 8 contained a Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (EFLNRWIT; **pep3**; IL-2₁₃₆₋₁₄₃) octa-peptide (SEQ ID NO:1). These three peptides were synthesized, and their effects on IL-2-induced T cell adhesion to FN were studied. The Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1), inhibited, in a dose-dependent manner, the adhesion of IL-2-activated T cells to FN; the Arg-Met-Leu-Thr (SEQ ID NO:2) is apparently the most potent inhibitor (Fig. 4A). Maximum inhibition was achieved with about 0.1 pg/ml (0.2 pM) for Arg-Met-Leu-Thr (SEQ ID NO:2), and 1 pg/ml of either Ile-Val-Leu or Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) (2.91 and 0.92 pM, respectively). The inhibitory dose-response curves

of Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) are similar to those of the HPLC fractions 2 and 8, respectively, from which they were derived.

To examine the specificity, on the biologic and chemical levels, of the inhibition of T cell adhesion by the elastase-generated, synthetic IL-2 peptides, we synthesized the three IL-2 peptides in their inverse amino acid sequences, Leu-Val-Ile, Thr-Leu-Met-Arg (SEQ ID NO:38), and Thr-Ile-Trp-Arg-Arg-Asn-Leu-Phe-Glu (SEQ ID NO:39), and then tested their effects on IL-2-induced T cell adhesion to FN. The results, shown in Fig. 4B, indicate that none of these peptides, tested in a broad range of dosages, interfere with T cell adhesion. Thus, the anti-adhesive effects of Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides of IL-2 appear to be due to their direct biologic effect on responding lymphocytes.

Do the IL-2 peptides, Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) have to be present during the entire period of the assay to exert their inhibitory effects? The results, shown in Fig. 4C, indicate that most of the anti-adhesive effects of the three peptides persevered even if these peptides (at 1 pg/ml) were removed from the T cells prior to their activation with IL-2 and seeding onto the FN-coated surfaces. Apparently, their prolonged inhibitory potential may involve active intracellular signaling pathways. These results suggest that the IL-2 peptides neither exert their inhibitory activities on T cell adhesion to FN via binding to the ECM protein, nor to FN-specific β_1 integrins expressed on the adhering T cells.

Example 4

Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2) and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) inhibit T cell adhesion to LN, CO-IV and FN. The next experiment was designed and performed to verify that the three IL-2-derived peptides indeed affect T cell interactions with ECM glycoproteins other than FN. T cells were pre-exposed to the three peptides (at 10 pg/ml), and then activated with IL-2. The treated cells were then added to microtiter wells coated with CO-IV, LN, and FN. The results, shown in Fig. 5, indicate that both Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) inhibit T cell adhesion to the three major cell-adhesive glycoproteins of the ECM (Fig. 5),

suggesting that the elastase-generated IL-2 peptides exert their inhibitory effects over different subsets of β_1 integrins.

Example 5

5 | *Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) inhibit T cell chemotactic*
migration induced by IL-2 or MIP-1 β . Immune cell migration is the outcome of a subtle
biological equilibrium existing between adhesion and detachment events. Lymphocyte adhesion
to the subendothelial ECM and subsequent migration are two active processes that can overlap,
but are not mutually dependent events. Adhesion and migration may depend on the ability of the
10 | T cells to continuously integrate different pro- and anti-adhesive signals via their versatile
receptors for ECM, chemokines, cytokines, and possibly, also antigenic moieties. Therefore, we
next examined the effects of the Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-
Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides (at 1 pg/ml) on the IL-2- and MIP-1 β -
induced T cell chemotaxis through FN-coated polycarbonate membranes. The gradient
15 | generated by MIP-1 β and IL-2, which were placed in the lower compartment of the 48-well
chemotaxis apparatus, induced a marked T cell migration through FN-coated membranes, which
was about 3 to 4-fold higher than the control (Fig. 6). Both Ile-Val-Leu, Arg-Met-Leu-Thr
(SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) markedly ($p<0.01$)
inhibited T cell migration toward IL-2, by about 30, 90, and 60%, respectively. However,
20 | although the Ile-Val-Leu peptide, and to a lesser degree also the Arg-Met-Leu-Thr (SEQ ID
NO:2) peptide, markedly inhibited (80% and 60%, respectively) T cell chemotaxis toward MIP-
1 β , the Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) showed only a limited inhibitory
effect on the chemokine-induced T cell chemotaxis. Thus, in addition to the capacity of the Ile-
Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides to inhibit T cell-ECM
25 | adhesion, they seem to inhibit T cell migration through FN in response to a diffusible gradient
produced by IL-2 or MIP-1 β .

Example 6

Inhibition by the Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides of T cell adhesion to FN induced by various pro-inflammatory mediators and by mAb specific for molecules expressed on T cells. The preceding chemotaxis experiments indicated that the

5 | Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides can inhibit T cell adhesion and migration through FN barriers induced not only by IL-2, but also by the chemokine MIP-1 β . Therefore, in an attempt to further understand the possible physiologic relevance of such phenomena, we examined the ability of these peptides to inhibit the adhesion to FN of T cells stimulated by modes other than IL-2. At 10 pg/ml, both Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) and Ile-Val-Leu inhibited T cell adhesion to FN that was induced by various stimulators of T cells and modulators of the β_1 integrin functions tested (Fig. 7).

10 | However, at 0.1 pg/ml, Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) did not inhibit the PMA- and 8A2-induced adhesion, and the Ile-Val-Leu peptide did not inhibit PMA-, 8A2-, and anti-CD3 mAb-induced T cell adhesion to FN, which indicates that these modes of activation are less susceptible to IL-2-derived peptide-induced suppression than IL-2-mediated activation. In experiments similar to those shown in Figs. 7A and 7B, the control peptides (corresponding inverse sequences) did not affect T cell adhesion to FN induced by the indicated activators (data not shown). Hence, Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) and Ile-Val-Leu apparently inhibit T cell adhesion to the FN component of ECM via a common intracellular event that is linked to the regulation of the avidities and affinities of β_1 integrins, and therefore, to their ligand recognition and binding.

Example 7

25 | *Inhibition of the reorganization of the actin cytoskeleton in FN-attached activated T cells by the Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptide.* The adhesion of immune cells to ECM is dependent on the sequestering of the cytoplasmic domains of integrins in focal adhesion sites, together with actin-containing microfilament bundles (Sanchez-Mateos et al., 1996). Therefore, we examined the effect of Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) on the morphologies of adherent T cells. The T cells were activated with IL-2 or PMA, treated with the IL-2 peptides, and seeded onto FN-coated coverslips. After incubation and

fixation, the actin cytoskeleton of attached T cells was stained with TRITC-conjugated phalloidin. The morphologies of FN-bound IL-2- and PMA-activated T cells (Figs. 8A, 8B) were markedly different from those of non-activated lymphocytes (Fig. 8E); the activated T cells appeared spread, and their actin cytoskeleton performed distinct structures typical of ECM-adherent cells. The Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptide inhibited the redistribution of the actin skeleton in both the IL-2- (Fig. 8C) and PMA-treated (Fig. 8D) FN-adherent T cells. Control peptides (reverse sequences of each peptide **pep1**, **pep2**, **pep3**) did not inhibit the actin reorganization of the activated T cells (not shown). Hence, the adhesion-inhibiting activity of the IL-2-derived peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1), similar to the Ile-Val-Leu peptide (data not shown), appears to involve inhibition of the redistribution of the actin cytoskeleton, and therefore, changes in cell shape and spreading.

Example 8

The effect of synthetic IL-2 derived peptides and derivatives thereof on spontaneous cytokine (IL-8) secretion from intestinal epithelial cells. Human colonic epithelial cell lines, such as HT-29, have been shown to secrete pro-inflammatory cytokines such as IL-1 β and IL-8, in response to cytokines such as TNF- α , IL-1 β , IFN- γ , and to LPS (Ecknamm et al., 1993), or as a response to bacterial invasion (Jung et al., 1995).

The effect of the synthetic IL-2 peptides **pep1**, **pep2**, **pep3**, and other synthetic derivatives of the sequences shown in Table 1 below, was tested on spontaneous IL-8 secretion from HT-29 cells as follows: HT-29 cells (10^6 cells/well) were seeded and incubated in culture medium for 24 h at 37°C in 24-well plates with the desired peptide (1 nM), the supernatants were collected and IL-8 levels were determined by an ELISA assay, using Pharmingen antibody pairs, according to the manufacturer's instructions. The results in Table 1 show the O.D. readings of the ELISA assay (at 405 nm) as well as the percentage of inhibition of IL-8 secretion. A level of inhibition of about 30% or higher indicates that the peptide has relevant anti-inflammatory activity. These results show that the peptides inhibit the spontaneous secretion of IL-8 from a human colonic epithelial cell line and are, therefore, good candidates for the treatment of inflammatory, particularly, bowel diseases.

Table 1: Inhibitory effect of pep1, pep2, pep3 and analogues thereof on spontaneous IL-8 secretion from intestinal epithelial cells

Peptide	IL-8 secretion	
	Average O.D.	% inhibition
Background	71±1.4	
Medium alone	488±8.5	0
(pep1) Ile-Val-Leu,	54±19.1	104
(pep4) Asn-Ile-Asn-Val-Ile-Val-Leu (SEQ ID NO:3),	34±3.5	109
(pep5) Ile-Val-Leu-Glu-Leu-Lys-Gly (SEQ ID NO:4),	35±0.7	109
(pep6) Asn-Val-Ile-Val-Leu (SEQ ID NO:5)	100±14.8	93
(pep7) Ala-Val-Leu	170±8.5	76
(pep8) Ile-Ala-Leu	35±4.9	109
(pep9) Ile-Val-Ala	100±9.9	93
(pep10) Glu-Val-Leu	125±19.1	87
(pep2) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)	36±4.2	108
(pep15) Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:8)	33±0.7	109
(pep16) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys (SEQ ID NO:9)	126±7.1	87
(pep17) Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:10)	150±6.4	81
(pep18) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser (SEQ ID NO:11)	162±12.0	78
(pep19) Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:12)	152±30.4	81
(pep20) Arg-Trp-Ile-Thr (SEQ ID NO:13)	191±4.9	71
(pep21) Glu-Phe-Leu-Asn (SEQ ID NO:14)	195±14.1	70

(pep22) Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:15)	158±4.2	79
(pep23) Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:16)	36±14.1	108
(pep24) Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:17)	187±16.3	72
(pep25) Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:18)	172±2.8	76
(pep26) Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:19)	156±27.6	80
(pep27) Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr (SEQ ID NO:20)	309±18.4	43
(pep28) Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr (SEQ ID NO:21)	120±8.5	88
(pep29) Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr (SEQ ID NO:22)	139±19.8	84
(pep30) Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr (SEQ ID NO:23)	123±9.2	88
(pep31) Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr (SEQ ID NO:24)	112±18.4	90
(pep32) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala (SEQ ID NO:25)	80±24.0	98
(pep33) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-NH ₂ (SEQ ID NO:26)	73±5.7	100
(pep3) Arg-Met-Leu-Thr (SEQ ID NO:2)	44±0.7	107
(pep36) Ala-Met-Leu-Thr (SEQ ID NO:28)	201±17.7	69
(pep37) Arg-Ala-Leu-Thr (SEQ ID NO:29)	222±24.0	64
(pep38) Arg-Met-Ala-Thr (SEQ ID NO:30)	157±1.4	79
(pep39) Arg-Met-Leu-Ala (SEQ ID NO:31)	160±21.9	79
(pep40) Lys-Met-Leu-Thr (SEQ ID NO:32)	179±17.7	74
(pep41) Arg-Val-Leu-Thr (SEQ ID NO:33)	132±17.0	85
(pep42) Arg-Met-Leu-Thr-NH ₂ (SEQ ID NO:34)	226±28.3	63
(pep43) Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr (SEQ ID NO:35)	57±12.7	103
(pep44) Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr (SEQ ID NO:36)	307±29.7	43

5 The effect of the peptides was also tested on the TNF- α induced secretion of IL-1 β from intestinal epithelial cells. Test cells (HT-29 or Caco-2) were grown as confluent monolayers in 24-well tissue culture plates. After the cells reached confluence, the culture medium was changed and the cells (10⁶ cells/well) were incubated for 24 h at 37°C with the IL-2 peptides (or

control and derivative peptides) in 24-well plates with the addition of TNF- α . The peptides were added to the cells 1 hour prior to the addition of TNF- α . Following culture, the supernatants were harvested and analyzed for IL-1 β secretion by ELISA using a commercially available kit (Genzyme), according to the manufacturers' protocols. The results are similar to those obtained above, the peptides being shown to inhibit TNF- α induced secretion of IL-1 β in similar levels as above (data not shown).

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ANTI-INFLAMMATORY PEPTIDES DERIVED FROM IL-2 AND ANALOGUES
THEREOF

REFERENCE TO RELATED APPLICATIONS

- 5 The present application is the national stage under 35 U.S.C. 371 of international application PCT/IL99/00448, filed 19 August 1999, which designated the United States, which international application was published under PCT Article 21(2) in English.

Field of the Invention

- 10 The present invention relates to anti-inflammatory peptides derived from pro-inflammatory IL-2, derivatives thereof, and to pharmaceutical compositions comprising said anti-inflammatory peptides.

Background of the Invention

- 15 The migration of T cells through tissues is regulated by adhesion receptors, such as integrins, and by receptors that receive signals provided by pro-inflammatory mediators, such as cytokines, chemokines, and extracellular matrix (ECM)-degrading enzymes (Gilat et al., 1996; Butcher and Picker, 1996).

- 20 IL-2 is a 15.5 kDa glycoprotein that participates in the development of inflammation and in the regulation of apoptosis (Taniguchi and Minami, 1993). In addition to its pro-activatory and proliferative roles, IL-2 also induces neutrophil adhesion to umbilical vein endothelial cells in a CD18-mediated manner (Li et al., 1996) as well as chemotactic responses in T cells, both directly and via regulating their expression of CC chemokine receptors (Loetscher et al., 1996). The IL-2
25 receptor consists of three distinct membrane chains: the α -, β - and γ -chains. Anti-IL-2 antibodies that recognize amino acid epitopes in the N-terminal region of IL-2 can inhibit IL-2-induced lymphocyte proliferation. The C-terminal portion of IL-2 and its three Cys residues seem to contribute to the folding and active conformation of IL-2 (Kuo and Robb, 1986).

- 30 The majority of neutrophil elastase (also termed human leukocyte elastase; HLE), which exists as either a membrane-bound or soluble moiety, is produced and released by neutrophils, although small amounts are also produced by macrophages, monocytes, and T cells (Packard et al., 1995).

Elastase degrades basement membrane and ECM glycoproteins, such as elastin, collagen, and fibronectin (FN), as well as molecules expressed on the surface of T cells, e.g., CD4, CD8, and CD2 (Doring et al., 1995).

5 **Summary of the Invention**

It has now been surprisingly observed, according to the present invention, that enzymatic degradation of IL-2 by proteolytic enzymes that participate in the breakdown of the extracellular matrix (ECM) results in small peptidic fragments that possess anti-inflammatory activity.

10 A first aspect of the present invention therefore relates to anti-inflammatory peptides derived from IL-2, and to anti-inflammatory derivatives of said peptides.

The anti-inflammatory peptides of the present invention are defined as such by virtue of their ability to inhibit at least one of the following processes in vitro: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-
15 induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells.

20 A second aspect of the present invention relates to a method for identifying anti-inflammatory peptides derived from IL-2, which comprises:

(i) carrying out enzymatic digestion of IL-2 with a proteolytic enzyme that participates in the breakdown of the extracellular matrix (ECM);

(ii) testing the fractions obtained in (i) for their in vitro ability to inhibit at least one of the
25 following processes: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells;

(iii) selecting the fractions of (ii) active in at least one of the bioassays (a) to (e),
30 fractionating each fraction to isolate individual peptides thereof, and submitting each isolated peptide to sequencing and synthesis; and

(iv) carrying out one or more of the bioassays (a) to (e) with a peptide isolated in step (iii), and selecting those peptides that exhibit anti-inflammatory activity in at least one of said bioassays.

- 5 The second aspect of the invention further relates to the peptides obtainable from the method of identification described above and peptide derivatives thereof.

The proteolytic enzyme is one capable of processing the glycoprotein constituents of tissues and blood vessel walls and may be elastase, a collagenase or a metalloprotease such as metalloelastase, matrix metalloprotease-2 (MMP-2) and matrix metalloprotease-9 (MMP-9). In a preferred embodiment, the proteolytic enzyme is elastase.

The anti-inflammatory synthetic peptides of the present invention have been characterized by their possession of inhibitory activity in at least one of processes (a) to (e) given above, the anti-inflammatory peptide being preferably active in at least one of assays (a), (b) or (e).

A third aspect of the invention relates to pharmaceutical compositions comprising at least one anti-inflammatory synthetic peptide of the invention and a pharmaceutically acceptable carrier. This aspect includes also the use of said peptides for the preparation of pharmaceutical compositions for the treatment and/or alleviation of acute and chronic inflammatory disorders.

A fourth aspect of the invention relates to a method for the treatment and/or alleviation of acute and chronic inflammatory disorders comprising administering to a subject in need thereof an effective amount of an anti-inflammatory synthetic peptide of the invention.

Brief Description of the Drawings

Figs. 1A-1B show induction of T cell adhesion to fibronectin (FN), laminin (LN), collagen type IV (CO-IV) (Fig. 1A) and ECM (Fig. 1B) by IL-2. [⁵¹Cr]-Labeled human T cells were seeded onto FN-, LN-, CO-IV-, and ECM-coated microtiter wells together with IL-2. After 30 min at 37°C, non-adherent T cells were removed, the adherent cells were lysed, and the percentage of T cells that had adhered was determined. One experiment representative of four.

Fig. 2 depicts a chromatogram of IL-2 fractions obtained upon elastase proteolysis. IL-2 (1mg/ml) was incubated (12 hr, 37°C) with neutrophil elastase (50µg/ml in PBS). The resulting enzymatic digests were purified by HPLC. Fraction 1 consisted of salts used for the separation procedure. One experiment representative of three.

Fig. 3 shows effects of IL-2 fractions, generated by elastase degradation, on the IL-2-induced adhesion of T cells to FN. IL-2 (10 U/ml)-stimulated [⁵¹Cr]-labeled T cells were seeded, in the presence or absence of elastase-generated IL-2 protein products, onto wells coated with FN. After 30 min at 37°C, non-adherent T cells were removed, and the percentage of adhered cells was determined. One experiment representative of six.

Figs. 4A-4C show specific inhibition, by elastase-generated fractions 2 and 8 of IL-2 and by their synthetic peptides, of IL-2-induced T cell adhesion to FN. Fig. 4A: T cell adhesion to FN in the presence of IL-2 fractions and the IL-2 peptides Ile-Val-Leu (IVL), Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (EFLNRWIT) (SEQ ID NO:1), and Arg-Met-Leu-Thr (RMLT) (SEQ ID NO:2), herein designated **pep1**, **pep2** and **pep3**, respectively. Fig. 4B. The effects on T cell adhesion to FN of the inversely synthesized IL-2 peptides Leu-Val-Ile (LVI), Thr-Leu-Met-Arg (TLMR) (SEQ ID NO:38) and Thr-Ile-Trp-Arg-Asn-Leu-Phe-Glu (TIWRNLFE) (SEQ ID NO:39). T cells were activated with IL-2 (10 U/ml) and seeded onto FN-coated wells in the presence of IL-2, fraction 2, fraction 8, or inversed IL-2 peptides. Fig. 4C. Effects of pre-treatments of T cells with the IL-2 peptides **pep1**, **pep2** and **pep3**, on the subsequent IL-2-induced T cell adhesion to FN. T cells used were untreated or pre-treated with the indicated peptides (1 pg/ml; 60 min, 37°C, 10% CO₂, humidified atmosphere), washed twice, exposed to IL-2, and seeded onto the FN-coated wells. After 30 min at 37°C, T cell adhesion was measured. One experiment representative of five.

Fig. 5 shows inhibition by the IL-2 peptides **pep1**, **pep2** and **pep3**, of T cell adhesion to LN, CO-IV and FN. T cells were pre-treated with the indicated IL-2 peptides (10 pg/ml, 30 min, 37°C, 10% CO₂, humidified atmosphere) and then with IL-2 (10 U/ml). The T cells were then

seeded onto microtiter wells that were pre-coated (1 µg/well) with the various ECM glycoproteins. T cell adhesion was measured 30 min later.

Fig. 6 shows inhibition of IL-2-induced T cell migration through FN by **pep1** and **pep3**. T cells were pre-treated with the IL-2 peptides (1 pg/ml) or buffer alone and then placed in the upper wells of a chemotaxis chamber, in which IL-2 (10 U/ml) or MIP-1β (10 ng/ml) had been added to the lower compartment. T cell migration towards the chemotactic sources was assessed after 2 hr. One experiment representative of four.

Figs. 7A-7B show inhibition by **pep2** (Fig. 7A) and **pep1** (Fig. 7B) of T cell adhesion to FN, induced by various activators. Labeled T cells were seeded onto FN-coated wells in the presence of IL-2 (10 U/ml), IL-7 (50 ng/ml), MIP-1β (20 ng/ml), PMA (50 ng/ml), 8A2 mAb (1 µg/ml), or anti-human CD3 mAb (1 µg/ml). The IL-2-derived peptides, **pep1** or **pep2**, were also present in some wells. After 30 min at 37°C, non-adherent T cells were removed by washing, the remaining adherent cells were lysed, and the percentage of T cells that had adhered was determined. One experiment representative of four.

Figs. 8A-8E show inhibition by **pep2** of the spreading and redistribution of the actin cytoskeleton in IL-2- and PMA-activated FN-adherent T cells. T cells were activated (48 hr) with IL-2 (50 U/ml). The T cells were then washed and seeded onto FN-coated coverslips, in medium alone (8E), or in the presence of IL-2 (100 U/ml; 8A and 8C), PMA (50 ng/ml; 8B and 8D), or **pep2** (10 pg/ml; 8C and 8D). After incubation, the intracellular actin filaments of the fixed FN-attached T cells were stained. Original magnification, x1000.

Detailed Description of the Invention

IL-2 and elastase are prominent mediators of leukocyte extravasation and migration from the vasculature through the ECM to sites of inflammation. The present invention is based on the assumption that, in contrast to the pro-adhesive effects of the intact IL-2 molecule, certain short IL-2-derived peptides, which may occur in vivo, can inhibit the interactions of T cells with ECM, and that this interference is independent of the effects of the peptides on the activation of

T cells by IL-2. We also assumed that such moieties of IL-2 can prevent the arrival of T cells at inflamed sites.

An important feature of elastase is its ability to act in both soluble and immobilized forms, since a migrating immune cell that expresses immobilized elastase may encounter matrix-bound IL-2, among other cytokines. This made neutrophil elastase a likely candidate for the physiologic production of these inhibitory peptides. Indeed, it was found, according to the present invention, that the processing of recombinant IL-2 by elastase resulted in the production of at least eight different by-products.

Three of the products generated by processing of recombinant IL-2 by neutrophil elastase (present in HPLC fractions 2, 7 and 8), inhibited IL-2-mediated T cell adhesion to FN. Amino acid composition analysis and amino acid sequencing revealed that fraction 2 contained the tripeptide Ile-Val-Leu (IL-2₁₁₂₋₁₁₄) (herein designated **pep1**) and the tetrapeptide Arg-Met-Leu-Thr (IL-2₅₈₋₆₁) (SEQ ID NO:2) (herein designated **pep3**), and fraction 8 the octapeptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (IL-2₁₃₆₋₁₄₃) (SEQ ID NO:1) (herein designated **pep2**). The **pep3** appeared to be located within the IL-2-binding site of the α -chain of the IL-2R, whereas **pep1** and **pep2** are located at sites far from the receptor-binding sites of IL-2. These peptides, at a picomolar range of concentrations (i.e., 0.01-1 pg/ml), inhibited the IL-2, as well as MIP-1 β -induced chemotaxis of human T cells through FN-coated polycarbonate membranes.

These peptides were synthesized and the synthetic peptides were shown to exhibit the same activity as the IL-2 digested fractions 2 and 8. Thus, in one preferred embodiment, the IL-2 derived peptides of the invention are the peptides:

- (**pep1**) Ile-Val-Leu,
- (**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)
- (**pep3**) Arg-Met-Leu-Thr (SEQ ID NO:2),

The chemoattractive capacity of IL-2 in T cell migration studies in vitro has been shown by using bare polycarbonate filters or collagen- or Matrigel-coated membranes as immobilized

substrates. T cell migration in these systems was proved to be IL-2R β -chain-specific and depended on the activities of the matrix-degrading gelatinases (Leppert et al., 1995). According to the present invention, the IL-2 peptides **pep1**, **pep2**, **pep3**, in addition to their anti-migratory effects, also inhibited T cell adhesion to FN induced by various physiologic and non-physiologic stimuli. Nevertheless, none of them, at 1 to 100 pg/ml, interfered with either PHA- or IL-2-mediated proliferative responses of human T cells, nor did these peptides inhibit the secretion of TNF- α and IFN- γ from these proliferating cells (not shown). Moreover, the inversely synthesized molecules, Leu-Val-Ile, Thr-Leu-Met-Arg (SEQ ID NO:38), and Thr-Ile-Trp-Arg-Asn-Leu-Phe-Glu (SEQ ID NO:39), did not inhibit T cell adhesion to FN. Thus, the migration- and adhesion-suppressive capabilities of **pep1**, **pep2** and **pep3** are specific, and are not due to toxic cell death.

How do the elastase-derived IL-2 peptides exert their inhibitory functions? We have demonstrated, according to the present invention, that the three peptides **pep1**, **pep2** and **pep3** do not have to be present during the entire period of the adhesion assay, since their anti-adhesive effect was apparent even after their removal from the assay prior to T cell activation with IL-2. This finding also implies that **pep1**, **pep2** and **pep3** do not function by interacting with putative cell-adhesive epitopes present on the tested ECM glycoproteins. It seems to be highly unlikely that two of these peptides (**pep1** and **pep2**) exert their biologic functions by interacting with the IL-2R subunits or by directly binding to β_1 -specific integrins. The IL-2 peptides interfered with different modes of T cell activation, leading to their adhesion to the tested matrix proteins. Moreover, both **pep1** and **pep2**, used in a picomolar range of concentrations, appear to block T cell adhesion not only to FN, mediated predominantly via the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, but also to LN; T cell adhesion to LN was mediated primarily through the $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins. Interesting, however, is the fact that **pep3** (Arg-Met-Leu-Thr (SEQ ID NO:2)) resides within the IL-2R α -binding site of IL-2; two residues, Arg⁵⁸ and Phe⁶², which are present within and adjacent to this peptide, respectively, were shown to be critical for IL-2-IL-2R interactions. Therefore, it will also be interesting to examine whether the degradation of IL-2 by elastase produces compounds that can interfere with IL-2 binding to its receptor and with the biologic outcome of such molecular interactions.

The ability of **pep2** as well as **pep1** and **pep3** (data not shown) to inhibit the PMA- and IL-2-induced T cell adhesion to FN is probably linked to its ability to block the reorganization of the intracellular actin cytoskeleton. Integrin-cytoskeleton associations can modulate cell adhesion to ECM ligands, cell spreading in areas of cell contact with the substratum, and the micro-clustering and redistribution of β_1 integrins on the cell surface at sites of focal adhesion located at the ends of the actin fibers (Otey et al., 1990). Similar observations were noted for T cell activation by IL-2 (and PMA) and attachment to FN. Although the intracellular mechanisms of action of the elastase-generated fractions and peptides have not yet been determined, we postulate that these proteins effect the adhesion and migration of T cells within the ECM by active inhibition of intracellular signal transduction pathways linked to cytoskeleton organization, resulting in an inhibition of micro-clustering and an association of integrins with cytoskeletal elements.

Our findings according to the present invention imply that the tissue-invading T cells themselves can dynamically regulate their own functions. Both adhesion- and migration-promoting stimuli (i.e. intact IL-2) and suppressive by-products of inflammatory mediators can be present, although not necessarily simultaneously, within the inflammatory milieu. At the early stages of inflammation, both IL-2 and elastase may function concomitantly to activate T cells to penetrate tissues. Later, the degradation peptide products of IL-2, generated by elastase, may inhibit T cell migration, inhibit the co-stimulatory effects of IL-2 and other mediators, and probably signal the termination of the inflammatory reaction.

As stated before, the invention relates to anti-inflammatory peptides derived from the IL-2 sequence and to derivatives thereof, said anti-inflammatory activity being assessed by their ability to inhibit at least one of the following processes in vitro: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells.

The in vitro bioassays (a) to (e) are well-known in the art. Thus, for example, assays (a), (b) and (e) may be carried out as described in the section Materials and Methods hereinafter. Assay (c), for inhibition of cytokine - or mitogen-induced T cell proliferation, is carried out as follows: A total of 25×10^5 of human T cells in 0.2 ml of RPMI medium supplemented with 5% heat-inactivated FCS (Gibco), antibiotics, 1 % Hepes buffer, 1% glutamine and 5×10^{-5} M 2-mercaptoethanol (ME), are added per well. T cell activation is tested by adding either the cytokine IL-2 (100 U/ml) or the mitogen Con-A (2.5 μ g/ml) to the culture wells in the beginning of the assay. The IL-2-derived peptides or their derivatives are added to the culture wells (different amounts, 10 microliters) for the entire period of the assay. [3 H]-Thymidine is added to the microtiter 96 U-form well plates 18h before harvesting the cultures on day 3 onto glass fiber filters and counted by liquid scintillation counter. Mean CPM and SD from quadruplicate culture are then presented. Assay (d), for inhibition of cytokine secretion by cytokine- or mitogen-stimulated T cells, is carried out similar to the proliferation assay (c), except for one modification: the supernatants of activated T cells (or any other leukocytes derived from human blood), treated with the different peptides, are collected and assayed for IL-2 and TNF- α , using conventional ELISA kits.

In vivo bioassays may also be used to determine anti-inflammatory activity of the peptides of the invention, examples of which include: (1) inhibition of experimental delayed type hypersensitivity (DTH) reactivity carried out, for example, as described in PCT Publication No. WO 94/11006, Example 5.2; (2) treatment of experimental adjuvant arthritis (AA) in rats carried out, for example, as described in PCT Publication No. WO 94/11006, Example 5.7: and (3) treatment of experimental autoimmune encephalomyelitis (EAE) in guinea pigs, a model disease for multiple sclerosis, carried out, for example, as described in US Patent No. 5,206,223.

The term "peptides of the invention" as used hereinafter includes the synthetic peptides **pep1**, **pep2**, and **pep3** derived from IL-2 as well as synthetic analogues thereof, and comprise:

(i) peptides **pep1**, **pep2**, and **pep3** of the sequences:

(**pep1**) Ile-Val-Leu

(**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)

(**pep3**) Arg-Met-Leu-Thr (SEQ ID NO:2)

(ii) peptides obtained from **pep2** by deletion of one or more amino acid residues;

(iii) peptides obtained by addition to peptides (i) or (ii) of one or more natural or non-natural amino acid residues;

(iv) peptides obtained by replacement of one or more amino acid residues of peptides (i) to (iii) by the corresponding D-stereomer, by another natural amino acid residue or by a non-natural amino acid residue;

(v) chemical derivatives of the peptides (i) to (iv);

(vi) cyclic derivatives of peptides (i) to (v);

(vii) dual peptides consisting of two of the same or different peptides (i) to (vi), wherein the peptides are covalently linked to one another directly or through a spacer; and

(viii) multimers comprising a number of the same or different peptides (i) to (vi).

The term “peptide derivative” or “peptide analogue” as used throughout the specification and claims herein is intended to include the derivatives defined in (ii) to (viii) above, namely peptides obtained by deletion, addition, or replacement of a natural amino acid residue by the corresponding D-stereomer or by a non-natural amino acid residue, chemical derivatives of the peptides, cyclic peptides, dual peptides and multimers of the peptides.

Typically, modifications are made that retain the anti-inflammatory effect of the parent peptides **pep1**, **pep2** and **pep3**. Any of the above modifications may be utilized alone or in combination, provided that the modified sequence retains anti-inflammatory activity, preferably at least 30% of the anti-inflammatory activity, more preferably at least the same activity or higher, of the parent peptide, identified by means of the above described assays.

Deletion of 1-4 amino acid residues of **pep2** may be made at the N- or the C-terminal such as for example peptides **pep19** and **pep21**, respectively, in Table 1 hereinafter. Addition of one or more natural or non-natural amino acid residues may be made at the N- or the C-terminal of each of the parent peptides **pep1**, **pep2** and **pep3**, such as for example peptides **pep4**, **pep5** and **pep6** (analogues of **pep1**), the peptides **pep15** and **pep17** (analogues of **pep2**), and the peptide **pep43** (analogue of **pep3**).

Substitutions include replacement of the natural amino acid residues by the corresponding D-amino acid residue, for example to increase blood plasma half-life of a therapeutically administered peptide, or by different natural amino acid residues or by non-natural amino acid residues. Thus, the peptide or peptide derivative of the invention may be all-L, all-D or a D,L-peptide. Examples of non-natural amino acids include, but are not limited to, N α -alkyl, particularly N α -methyl, amino acids*, C α -alkyl, particularly C α -methyl, amino acids*, halo derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*, p-Br-phenylalanine* and p-I-phenylalanine*, β -alanine (β -Ala)*, L- α -aminobutyric acid*, L- γ -aminobutyric acid (γ -Abu)*, L- α -aminoisobutyric acid (α -Aib)*, L- ϵ -aminocaproic acid*, 7-aminoheptanoic acid*, L-norleucine (Nle)*, L-norvaline (Nva)*, L-p-nitro-phenylalanine*, L-hydroxyproline (Hyp)*, L-thioprolin*, ornithine (Orn)**, homoarginine (homoArg)#, diaminobutyric acid (Dab)**, pyridylalanine*, thienylalanine*, naphthylalanine*, phenylglycine*, methyl derivatives of phenylalanine (Phe) such as L-4-methyl-Phe* and L-pentamethyl-Phe*, L-4-amino-Phe*, L-4-isopropyl-Phe*, L-4-phenyl-Phe*, L-4-benzyl-Phe*, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (L-Tic)*, L-methyl-Tyr*, and L-diaminopropionic acid (Dap)**. The notation * has been utilised to indicate the hydrophobic nature of the moiety whereas # has been utilised to indicate the hydrophilic nature of the derivative, and ** indicates amphipathic characteristics.

A "chemical derivative" of a peptide of the invention includes, but is not limited to, a derivative containing additional chemical moieties not normally a part of the peptide provided that the derivative retains the anti-inflammatory function of the peptide. Examples of such derivatives are: (a) N-acyl derivatives of the amino terminal or of another free amino group, wherein the acyl group may be either an alkanoyl group, e.g. acetyl, hexanoyl, octanoyl, or an aroyl group, e.g. benzoyl; (b) esters of the carboxy terminal or of another free carboxy or hydroxy groups; (c) amides of the carboxy terminal or of another free carboxy groups produced by reaction with ammonia or with a suitable amine, resulting in the C-terminus or another carboxy group being in the form -C(O)-NH-R, wherein R may be hydrogen, C1-6 alkyl, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl or hexyl, aryl such as phenyl, and aralkyl such as benzyl, such amidation being advantageous in providing additional stability and possibly enhanced activity to

the peptide; (d) glycosylated derivatives; (e) phosphorylated derivatives; (f) derivatives conjugated to lipophilic moieties e.g. caproyl, lauryl, stearoyl; and (g) derivatives conjugated to an antibody or other cellular ligands. Also included among the chemical derivatives are those derivatives obtained by modification of the peptide bond -CO-NH-, for example by (a) reduction to -CH₂-NH- ; (b) alkylation to -CO-N (alkyl)- ; (c) inversion to -NH-CO- .

The term "cyclic peptides" as used herein are cyclic derivatives containing either an intramolecular disulfide bond, i.e. -S-S-, an intramolecular amide bond, i.e. -CONH- or -NHCO-, or intramolecular S-alkyl bonds, i.e. -S-(CH₂)_n-CONH- or -NH-CO(CH₂)_n-S-, wherein n is 1 or 2. The cyclic derivatives containing an intramolecular disulfide bond may be prepared by conventional solid phase synthesis while incorporating suitable S-protected cysteine or homocysteine residues at the positions selected for cyclization such as the amino and carboxy terminals of the peptides, with the option of including spacing residues, such as (Ala)_n, (Gly)_n where n is from 1 to 4, or non-natural amino acids such as 6-aminocaproic acid, between the terminal residue and the linking residue. The linking residues may then be linked together using known techniques to form cyclized peptide derivatives. For example, the linear peptides Cys-Ile-Val-Leu-Ala-Cys (SEQ ID NO:6), Cys-Ile-Val-Leu-Ala-Ala-Cys (SEQ ID NO:7), Cys-Arg-Met-Leu-Thr-Ala-Cys (SEQ ID NO:37) and Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys (SEQ ID NO:27) were prepared and cyclized according to methods known in the art for formation of a disulphide bond. Following completion of the chain assembly, cyclization can be performed either by selective removal of the S-protecting groups with a consequent on-support oxidation of free corresponding two SH-functions, to form S-S bonds, followed by conventional removal of the product from the support and appropriate purification procedure, or by removal of the peptide from the support along with complete side-chain deprotection, followed by oxidation of the free SH-functions in highly dilute aqueous solution. The cyclic derivatives containing an intramolecular amide bond may be prepared by conventional solid phase synthesis while incorporating suitable amino and carboxyl side-chain protected amino acid derivatives at the positions selected for cyclization. The cyclic derivatives containing intramolecular -S-alkyl bonds may be prepared by conventional solid phase synthesis while incorporating an amino acid residue with a suitable amino-protected side chain, and a suitable S-protected cysteine or homocysteine residue at the positions selected for cyclization.

A “dual peptide” according to the invention consists of two the same or different peptides or peptide derivatives of the invention covalently linked to one another directly or through a spacer such as by a short stretch of alanine residues or by a putative site for proteolysis by cathepsin (see U.S. Patent No. 5,126,249 and European Patent No. 495,049 with respect to such sites). This will induce site-specific proteolysis of the preferred form into the two desired analogues.

“Multimers” according to the invention consist of polymer molecules formed from a number of the same or different peptides or derivatives thereof. The polymerization is carried out with a suitable polymerization agent, such as 0.1% glutaraldehyde (Audibert et al. (1981) Nature, 289 : 593)

Modifications to the amino acid residues in the peptides of the present invention are discussed below using nomenclature as given in the Table below:

ALIPHATIC	Non-polar, neutral	Gly, Ala, Pro
Low hydrophobic		
High hydrophobic		Ile, Leu, Val, Met
Low hydrophilic	Polar, neutral	Cys, Ser, Thr
High hydrophilic		Asn, Gln
Hydrophilic	Polar – negatively charged	Asp, Glu
	Polar – positively charged	Lys, Arg, His
AROMATIC hydrophobic		Phe, Trp, Tyr

Thus, in one preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Ile-Val-Leu (**pep1**), and to anti-inflammatory peptides resulting from the modification thereof by:

(a) elongation by up to 3-4 further amino acid residues at the N- and/or C-terminal, such as in **pep11** and **pep13**, or preferably according to the natural sequence of IL-2;

(b) substitution of the Ile residue by a natural or non-natural amino acid hydrophilic polar neutral or negatively charged, or hydrophobic non-polar neutral amino acid residue, preferably selected from Glu, Asp, Asn, Gln, Ala, Val;

(c) substitution of the Val residue by a hydrophobic, non-charged natural or non-natural amino acid residue, preferably selected from Ala, Ile, Leu, Met, Nle, Phe;

(d) substitution of the Leu residue by a hydrophobic, non-charged natural or non-natural amino acid residue, preferably selected from Ala, Ile, Met, Nle, Phe, Val;

(e) amidation of the C-terminal Leu residue,

(f) cyclization of **pep1** or of any peptide of (a) to (e); and

(g) any combination of (a) to (f).

Examples of such modified peptides derived from **pep1** include;

(**pep4**) Asn-Ile-Asn-Val-Ile-Val-Leu (SEQ ID NO:3),

(**pep5**) Ile-Val-Leu-Glu-Leu-Lys-Gly (SEQ ID NO:4),

(**pep6**) Asn-Val-Ile-Val-Leu (SEQ ID NO:5)

(**pep7**) Ala-Val-Leu

(**pep8**) Ile-Ala-Leu

(**pep9**) Ile-Val-Ala

(**pep10**) Glu-Val-Leu

(**pep11**, linear) and (**pep12**, cyclic) Cys-Ile-Val-Leu-Ala-Cys (SEQ ID NO:6) and,

(**pep13**, linear) and (**pep14**, cyclic) Cys-Ile-Val-Leu-Ala-Ala-Cys (SEQ ID NO:7).

In a further preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (**pep2**) (SEQ ID NO:1), and to anti-inflammatory peptides resulting from the modification thereof by;

(a) elongation by up to 4 further amino acid residues at the C and/or N terminal ends, such as in **pep34**, or preferably according to the natural sequence of IL-2;

(b) substitution of the Glu residue by a natural or non-natural charged or polar charged amino acid residue, preferably selected from Lys, Arg, Asp, Gln, Asn;

(c) substitution of the Phe residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(d) substitution of the Leu residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(e) substitution of the important Asn residue by a hydrophilic, non-charged, aliphatic natural or non-natural amino acid residue such as Gln;

(f) substitution of the Arg residue by a positively charged, natural or non-natural amino acid residue, preferably selected from Lys, Orn, homoArg;

(g) substitution of the Trp residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Ile, Leu, Nle, Tic, Phe, 4-phenyl-Phe, 4-methyl-Phe;

(h) substitution of the Ile residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Phe, Leu, Nle, Tic;

(i) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue preferably selected from Cys, Ser;

(j) truncation by up to 4 amino acid residues from either the C or N terminal;

(k) amidation of the C-terminal Thr;

(l) cyclization of **pep2** or of any peptide of (a) to (k); and

(m) any combination of (a) to (l).

Examples of such modified peptides derived from **pep2** include:

- | | |
|------------------|--|
| (pep15) | Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:8) |
| (pep16) | Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys (SEQ ID NO:9) |
| (pep17) | Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:10) |
| (pep18) | Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser (SEQ ID NO:11) |
| (pep19) | Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:12) |
| (pep20) | Arg-Trp-Ile-Thr (SEQ ID NO:13) |
| (pep21) | Glu-Phe-Leu-Asn (SEQ ID NO:14) |

(**pep22**) Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:15)
 (**pep23**) Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:16)
 (**pep24**) Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:17)
 ((**pep25**) Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:18)
 5 (**pep26**) Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:19)
 (**pep27**) Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr (SEQ ID NO:20)
 (**pep28**) Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr (SEQ ID NO:21)
 (**pep29**) Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr (SEQ ID NO:22)
 (**pep30**) Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr (SEQ ID NO:23)
 10 (**pep31**) Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr (SEQ ID NO:24)
 (**pep32**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala (SEQ ID NO:25)
 (**pep33**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-NH₂ (SEQ ID NO:26) and,
 (**pep34**, linear) and (**pep35**, cyclic) Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys
 (SEQ ID NO:27).

15 In a further preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Arg-Met-Leu-Thr (**pep3**), and to anti-inflammatory peptides resulting from the modification thereof by:

- (a) elongation by up to 4 further amino acid residues at the C and/or N terminal end,
 20 such as in **pep45**, or preferably according to the natural sequence of IL-2;
- (b) substitution of the Arg residue by a natural or non-natural positively charged amino acid residue, preferably selected from Lys, Orn, homoArg, diaminobutyric acid;
- (c) substitution of the Met residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Ile, Leu, Nle, Tic;
- 25 (d) substitution of the Leu residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Nle, Tic;
- (e) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue such as Ser, Cys;
- (f) amidation of the C-terminal Thr residue;
- 30 (g) cyclization of **pep3** or of any peptide of (a) to (f); and
- (h) any combination of (a) to (g).

Examples of such modified peptides derived from **pep3** include;

- (**pep36**) Ala-Met-Leu-Thr (SEQ ID NO:28)
- (**pep37**) Arg-Ala-Leu-Thr (SEQ ID NO:29)
- 5 (**pep38**) Arg-Met-Ala-Thr (SEQ ID NO:30)
- (**pep39**) Arg-Met-Leu-Ala (SEQ ID NO:31)
- (**pep40**) Lys-Met-Leu-Thr (SEQ ID NO:32)
- (**pep41**) Arg-Val-Leu-Thr (SEQ ID NO:33)
- (**pep42**) Arg-Met-Leu-Thr-NH₂ (SEQ ID NO:34)
- 10 (**pep43**) Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr (SEQ ID NO:35)
- (**pep44**) Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr (SEQ ID NO:36) and,
- (**pep45**, linear) and (**pep46**, cyclic) Cys-Arg-Met-Leu-Thr-Ala-Cys (SEQ ID NO:37).

The peptides and peptide derivatives of the invention are obtained by any method of peptide synthesis known to those skilled in the art, such as for example by solid phase peptide synthesis.

The present invention is also directed to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and at least one anti-inflammatory peptide or peptide derivative of the invention. The pharmaceutical composition will be administered according to known modes of peptide administration, including oral, intravenous, subcutaneous, intraarticular, intramuscular, inhalation, intranasal, intrathecal, intradermal, transdermal or other known routes. The dosage administered will be dependent upon the age, sex, health condition and weight of the recipient, and the nature of the effect desired.

The peptides of the invention for use in therapy are typically formulated for administration to patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. The formulation will depend upon the nature of the peptide and the route of administration but typically they can be formulated for topical, parenteral, intramuscular, intravenous, intraperitoneal, intranasal inhalation, lung inhalation, intradermal or intra-articular administration. The peptide may be used in an injectable form. It may therefore be mixed with any

vehicle which is pharmaceutically acceptable for an injectable formulation, preferably for a direct injection at the site to be treated, although it may be administered systemically.

The pharmaceutically acceptable carrier or diluent may be, for example, sterile isotonic saline solutions, or other isotonic solutions such as phosphate-buffered saline. The peptides of the present invention may be admixed with any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s). It is also preferred to formulate the peptide in an orally active form.

Tablets or capsules of the peptides may be administered singly or two or more at a time, as appropriate. It is also possible to administer the peptides in sustained release formulations.

Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient.

There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Alternatively, the peptides of the invention, can be administered by inhalation or in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. An alternative means of transdermal administration is by use of a skin patch. For example, they can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. They can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.

For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents. For such oral administration, the peptide may preferably be formed into microcapsules or nanoparticles together with biocompatible polymers such as poly-lactic acid and the like.

Co., St. Louis, MO); collagen type IV (CO-IV; ICN, Costa Mesa, CA); and HEPES buffer, antibiotics, heat-inactivated FCS, sodium pyruvate, DMEM, CSS and RPMI-1640 (Beit-Haemek, Israel). An anti- β_1 integrin-specific affinity-modulating mAb, 8A2, was donated by Dr. J. M. Harlan (Washington University, Seattle, WA). All protected amino acids, coupling
5 reagents, and polymers were obtained from Nova Biochemicals (Läufelfingen, Switzerland). Synthesis-grade solvents were obtained from Labscan (Dublin, Ireland). HPLC solvents and columns were obtained from Merck (Darmstadt, Germany).

Human colonic epithelial cell lines HT-29 (ATCC HTB38) and Caco-2 (ATCC HTB27) were
10 obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in culture medium (DMEM supplemented with 10% cosmic calf serum (CSS), 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin), at 37°C and atmosphere of 5% CO₂. Cell viability was assessed using the standard MTT method.

15 *T cell adhesion assays.* Human T cells were purified from the peripheral blood of healthy donors, and T cell adhesion to immobilized protein substrates was examined as follows: Human leukocytes were isolated on a Ficoll gradient, washed, and incubated (2 h, 37°C, 7.5% CO₂, humidified atmosphere) on petri dishes. The non-adherent cells were then collected and incubated (1 h, 37°C, 7.5% CO₂, humidified atmosphere) on nylon wool columns (Novamed
20 Ltd., Jerusalem, Israel). Unbound cells were eluted from the columns by extensive washings. The resulting cell population was always >92% T cells. Intact IL-2 or IL-2-derived peptides (in 50 μ l) were added to flat-bottom microtiter wells that had been pre-coated with ECM or ECM proteins (FN or LN; 1 μ g/well) and blocked with 0.1% BSA. After 4-6 h at 37°C, the wells were washed and ⁵¹[Cr]-labeled T cells were added to the wells, 10⁵ cells/100 μ l of adhesion
25 medium (RPMI-1640 supplemented with 0.1% BSA, 1% sodium pyruvate, 1% HEPES buffer). The microtiter plates containing the cells were incubated (30 min, 37°C) in a humidified, 7.5% CO₂ atmosphere, and then washed. The adherent cells were lysed, and the resulting supernatants were removed and analyzed in a γ -counter. For each experimental group, the results were expressed as the mean percentage \pm SD of bound T cells from quadruplicate wells. To some
30 wells, different concentrations of soluble IL-2 were added concomitantly with the T cells, and

The compositions (as well as the peptides alone) can also be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. In this case, the compositions will comprise a suitable carrier or diluent. For parenteral administration, the compositions are best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood.

For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The peptides and peptide derivatives of the invention are for use in the treatment of or amelioration of acute and chronic inflammatory disorders including, but not being limited to, autoimmune diseases such as rheumatoid arthritis, diabetes type I, multiple sclerosis, systemic lupus erythematosus, uveitis, bowel inflammation and Crohn's disease.

The present invention further relates to a method of treatment of a patient suffering from an inflammatory disorder which comprises administering to said patient an effective amount of a peptide or peptide derivative of the invention.

The invention will now be illustrated by the following non-limiting Examples.

EXAMPLES

Material and methods

Materials. The following reagents were obtained as indicated. Recombinant human IL-2 (sp. act. 18×10^6 U/mg; Chiron B.V., Amsterdam, The Netherlands); recombinant human IL-7 (sp. act. 2×10^5 U/mg; Immunex Corp., Seattle, WA); recombinant human macrophage inflammatory protein MIP-1 β (Pepro Tech, Rocky Hill, NJ); recombinant human TNF- α (Boehringer, Ingelheim, Germany); Affi-gel 10 beads (Pharmacia, NJ); fibronectin (FN) (Chemicon; Temecula, CA); BSA, laminin (LN), PMA, and TRITC-conjugated phalloidin (Sigma Chemical

with others, different concentrations of elastase-degraded IL-2-derived fractions, or the corresponding synthetic peptides, were added together with stimulators [PMA (50 ng/ml), IL-7 (50 ng/ml), MIP-1 β (20 ng/ml), 8A2 (1 μ g/ml), mAb anti-CD3 (1 μ g/ml), or IL-2 (10 U/ml)].

5 *Chemotaxis assays.* T cell chemotaxis was performed and analyzed as previously described (Ben-Baruch et al., 1997). Briefly, the migration of human T cells (0.5×10^6 cells in adhesion medium/well) was examined in a 48-well chemotaxis micro-chamber (Neuro-Probe Inc., Cabin John, MD). The two compartments of the micro-chambers were separated by a FN-coated polycarbonate filter (5- μ m pore size; Osmonics Protein Products, Livemore, CA). Where
10 indicated, MIP-1 β or IL-2 was added to the lower wells, and the T cells were added to the upper chambers together with the peptides. After incubation (120 min, 37°C, in a humidified, 7.5% CO₂ atmosphere), the filters were removed, fixed, and stained with a Diff-Quik staining kit (Dade, Düringen, Switzerland). The number of migrating T cells in five high-power fields (under 500x magnification; WILD Microscope, Heerbrugg, Switzerland) was evaluated. For
15 each group, the results are expressed as the mean number of cells in one high-power field.

Spontaneous or cytokine-induced secretion of IL-8 or IL-1 β from intestinal epithelial cell. Test cells (HT-29 or Caco-2) were grown as confluent monolayers in 24-well tissue culture plates. After the cells reached confluence, the culture medium was changed and the cells (10^6
20 cells/well) were incubated for 24 h at 37°C with the IL-2 peptides (or control and derivative peptides) in 24-well plates without (spontaneous) or with the addition of TNF- α (TNF- α -induced). When TNF- α was used, the peptides were added to the cells 1 hour prior to the addition of TNF- α . Following culture, the supernatants were harvested and analyzed for IL-8 or IL-1 β secretion by ELISA using commercially available kits for IL-8 (Pharmingen) and IL-1 β
25 (Genzyme), according to the manufacturers' protocols.

Purification of elastase and elastase digestion of IL-2. Neutrophils were isolated from the whole blood of a healthy donor by dextran sedimentation and Ficoll-Hypaque gradient centrifugation, as previously described (Yavin et al., 1996). Elastase was isolated by aprotinin-
30 Sepharose affinity chromatography, followed by carboxymethyl-cellulose ion exchange

chromatography, as developed by Baugh and Travis (Yavin et al., 1996; Baugh and Travis, 1976). The purified elastase, which was lyophilized and stored at -20°C until used, was biochemically checked to be entirely free from cross-contamination with cathepsin G (not shown). IL-2 was dissolved in distilled water to yield a 1mg/ml solution. Lyophilized neutrophil elastase (50 µg) was dissolved in 1 ml of PBS and immediately added to the IL-2 solution. The elastase-IL-2 mixture was incubated (12 h) at 37°C. Aliquots were removed and stored at -20°C until subjected to HPLC separation.

Reverse phase HPLC. Elastase digests of IL-2 were purified with a prepacked Lichrospher-100 RP-18 column (4x25 mm, 5 µm bead size), using a binary gradient formed with 0.1% trifluoroacetic acid (TFA) in H₂O (solution A) and 0.1% TFA in 75% acetonitrile in H₂O [(solution B) at $t=0$ min, B=3.5%, at $t=5$ min, B=3.5%, and then, the concentrations began to increase: at $t=60$ min, B=100% (i.e. 75% acetonitrile)]. The flow-rate was constant on 0.8ml/min. A Spectra-Physics SP8800 liquid chromatography system (Fremont, CA) equipped with an Applied Biosystems 757 (Foster City, CA) variable wavelength absorbency detector was used. The column effluents were monitored by UV absorbency at 220 nm, and the chromatograms were recorded on a ChromeJet integrator. Fractions that were 20% or more above valley levels were pooled, rotoevaporated to a minimal volume, and diluted with HPLC grade water. The rotoevaporation and dilution with water step was performed twice to remove residual TFA and acetonitrile.

Amino acid composition of the synthetic peptides and amino acid sequence analysis. Purified peptide solutions [≈ 40 µg of peptide in 40 µl, with 5 µg of norvaline (an unnatural amino acid) as an internal standard] were rotoevaporated, hydrolyzed (10°C, 22 h) in 6N HCl under vacuum, and analyzed with an amino acid analyzer (HP1090, Palo Alto, CA). An on-line pre-column ortho-phthalaldehyde (OPA)/9-fluorenylmethoxycarbonyl (F-moc) derivatization, combined with reverse phase chromatography, was used to determine the amino acid composition of the peptides and the total peptide yield. Without exception, all of the peptides yielded excellent analysis ratios of corresponding amino acids deviations from expected values of less than 10%. Analysis of the elastase-generated IL-2 fractions was performed using a Model 470A gas phase microsequencer. Phenylthiohydantoin amino acid derivatives were

separated on-line by reverse phase HPLC on a PTH C-18 column (2.1 x 220 mm) using a Model 120A analyzer (Applied Biosystems, CA).

Solid phase peptide synthesis. IL-2-derived peptides were prepared by conventional solid phase peptide synthesis, using an AMS-422 automated solid phase multiple peptide synthesizer (Abimed, Langenfeld, Germany). The Fmoc strategy was used for peptide chain assembly, according to the commercial protocol. In each reaction vessel, we used 12.5 μ mol of Wang resin containing the first covalently bound corresponding N-Fmoc C-terminal amino acid (typically, polymer loadings of 0.3-0.7 mmol/g resin were used). Fmoc deprotection was achieved by two consecutive treatments with 20% piperidine in dimethyl formamide, usually 10-15 min each at 22°C, depending on the length of peptide and the Fmoc-protected amino acid type. The protecting groups used for the side chain of the amino acids were tert-butyloxycarbonyl for Trp and Lys; trityl for Asn, Cys, Gln and His; tert-butylester for Asp and Glu; and tert-butylether for Ser, Tyr and Thr. Usually, coupling was achieved using two successive reactions (typically 20-45 min each at 22°C, depending on the length of peptide and amino acid derivative type) with 50 μ mol (4 eqv) of N-Fmoc-protected amino acid, 50 μ mol (4 eqv) of benzotriazole-1-yl-oxy-tris-pyrolidino-phosphonium hexafluorophosphate (PyBop) reagent, and 100 μ mol (8 eqv) of N-methylmorpholine were all dissolved in dimethylformamide (DMF). The peptide was cleaved from the polymer by reacting (2 h, 22°C) the resin with trifluoroacetic acid/H₂O/triethylsilane (90/5/5; v/v/v). The solution containing the crude, unprotected peptides was then cooled down to 4°C, precipitated with ether (4°C), and centrifuged (15 min, 3000 rpm, 4°C). The pellet was washed and centrifuged (x3) with ether, dissolved in 30% acetonitrile in H₂O, and lyophilized. The lyophilized material was reconstituted in double distilled water before use; only the stock solution, not the diluted material, was stored at -20°C.

Synthesis of cyclic peptides. The linear peptides **pep12**, **pep14**, **pep35**, **pep46** were cyclized as follows: 0.5-1.0 mg of SH-free peptide was dissolved in 1 ml of ammonium acetate buffer (0.1N, pH 7.0). A solution of K₃ [Fe(CN)₆] (1-5mM) in the same buffer was slowly added to the stirred peptide solution at room temperature, in a titrimetric fashion until a stable faint

yellowish color was apparent. The solution revealed a negative 5,5'-dithio-bis(2-nitrobenzoic acid (DTNB; Ellman's Reagent)-test for free SH. Lyophilization and purification by HPLC were then performed as usual.

5 *Staining of actin cytoskeleton.* T cells were incubated (18 h, 37°C, 7.5% CO₂, humidified atmosphere) in culture medium. IL-2 or PMA was added to the cell cultures, which were then incubated for 24 h. The T cells were then washed and seeded onto FN-covered coverslips in the presence of either PMA (50 ng/ml), IL-2 (100 U/ml), or IL-2 peptides (0.1 ng/ml). After 1 h at 37°C, the adherent cells were fixed (3 min) with paraformaldehyde (3%) and Triton X-100
10 (0.5%), washed, and fixed (20 min) again with paraformaldehyde (3%). The fixed adherent cells were washed, treated with TRITC-phalloidin, and washed again. Photographs (x1000 magnification) were then taken.

Example 1

15 *Induction of the adhesion of T cells to ECM, FN, LN, and CO-IV by IL-2.* We examined the ability of soluble IL-2 to induce adhesion of human T cells to ECM, FN, LN, and CO-IV. The results indicated that IL-2 induced T cell adhesion to FN, LN, and CO-IV (Fig. 1A), as well as to intact ECM (Fig. 1B). Note that the adhesion of T cells to LN induced by IL-2 was lower than that induced to the other ECM glycoproteins. When T cells were activated only with PMA,
20 45±4.4% of them adhered to immobilized ECM and ECM glycoproteins (not shown). IL-2-induced T cell adhesion to the ECM glycoproteins was inhibited by anti-human β_1 integrin mAbs (not shown), which suggests that the pro-adhesive effects of IL-2 were induced via cell surface-expressed integrins. However, under our experimental conditions, IL-2 did not alter the T cell surface expression of β_1 integrins (not shown). Thus IL-2, in addition to other pro-
25 inflammatory mediators, appears to regulate the adhesiveness of resting human T cells to immobilized ECM and ECM glycoproteins.

Example 2

30 *The effects of IL-2 fragments obtained by elastase degradation on the interaction of T cells with FN.* We have assumed that the degradation of IL-2 can occur in the inflamed milieu in

which both cytokines, such as IL-2, and proteolytic enzymes, such as neutrophil elastase, are present. We also hypothesized that, in contrast to the intact IL-2 molecule, certain portions of IL-2 can abrogate the adhesiveness of activated T cells to ECM ligands. Hence, elastase and soluble IL-2 were incubated together at physiologic conditions. HPLC analysis of the elastase-degraded IL-2 revealed at least 8 peaks of IL-2, each of which represented at least one low molecular weight protein fragment (Fig. 2).

Next, we examined the ability of the HPLC-purified IL-2 fractions, which were generated by elastase-degradation, to inhibit soluble IL-2-induced interactions of T cells with FN. We chose to investigate the major peaks of HPLC-purified, elastase-degraded fractions of IL-2. Fractions 2, 7, and 8 inhibited the adhesion of T cells to immobilized FN in a dose-dependent and statistically significant fashion, whereas fractions 4 and the HPLC buffer did not (Fig. 3). Thus, certain IL-2 fragments, obtained by neutrophil elastase-processing of the cytokine, can inhibit IL-2-induced adhesion of T cells to FN.

Example 3

The effect of synthetic peptides, with putative amino acid compositions corresponding to fractions 2 and 8, on IL-2-induced T cell adhesion to FN. Next, the primary sequence of fractions 2 and 8 were analyzed by gas phase chromatography, because these elastase-generated fractions of IL-2 appeared to contain adhesion-suppressive peptides. Our analysis revealed that fragment 2 contained an Ile-Val-Leu (IVL; **pep1**; IL-2₁₁₂₋₁₁₄) and an Arg-Met-Leu-Thr (RMLT; **pep2**; IL-2₅₈₋₆₁) peptide (SEQ ID NO:2), whereas fragment 8 contained a Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (EFLNRWIT; **pep3**; IL-2₁₃₆₋₁₄₃) octa-peptide (SEQ ID NO:1). These three peptides were synthesized, and their effects on IL-2-induced T cell adhesion to FN were studied. The Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1), inhibited, in a dose-dependent manner, the adhesion of IL-2-activated T cells to FN; the Arg-Met-Leu-Thr (SEQ ID NO:2) is apparently the most potent inhibitor (Fig. 4A). Maximum inhibition was achieved with about 0.1 pg/ml (0.2 pM) for Arg-Met-Leu-Thr (SEQ ID NO:2), and 1 pg/ml of either Ile-Val-Leu or Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) (2.91 and 0.92 pM, respectively). The inhibitory dose-response curves

of Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) are similar to those of the HPLC fractions 2 and 8, respectively, from which they were derived.

To examine the specificity, on the biologic and chemical levels, of the inhibition of T cell adhesion by the elastase-generated, synthetic IL-2 peptides, we synthesized the three IL-2 peptides in their inverse amino acid sequences, Leu-Val-Ile, Thr-Leu-Met-Arg (SEQ ID NO:38), and Thr-Ile-Trp-Arg-Asn-Leu-Phe-Glu (SEQ ID NO:39), and then tested their effects on IL-2-induced T cell adhesion to FN. The results, shown in Fig. 4B, indicate that none of these peptides, tested in a broad range of dosages, interfere with T cell adhesion. Thus, the anti-adhesive effects of Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides of IL-2 appear to be due to their direct biologic effect on responding lymphocytes.

Do the IL-2 peptides, Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) have to be present during the entire period of the assay to exert their inhibitory effects? The results, shown in Fig. 4C, indicate that most of the anti-adhesive effects of the three peptides persevered even if these peptides (at 1 pg/ml) were removed from the T cells prior to their activation with IL-2 and seeding onto the FN-coated surfaces. Apparently, their prolonged inhibitory potential may involve active intracellular signaling pathways. These results suggest that the IL-2 peptides neither exert their inhibitory activities on T cell adhesion to FN via binding to the ECM protein, nor to FN-specific β_1 integrins expressed on the adhering T cells.

Example 4

Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2) and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) inhibit T cell adhesion to LN, CO-IV and FN. The next experiment was designed and performed to verify that the three IL-2-derived peptides indeed affect T cell interactions with ECM glycoproteins other than FN. T cells were pre-exposed to the three peptides (at 10 pg/ml), and then activated with IL-2. The treated cells were then added to microtiter wells coated with CO-IV, LN, and FN. The results, shown in Fig. 5, indicate that both Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) inhibit T cell adhesion to the three major cell-adhesive glycoproteins of the ECM (Fig. 5),

suggesting that the elastase-generated IL-2 peptides exert their inhibitory effects over different subsets of β_1 integrins.

Example 5

5 *Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) inhibit T cell chemotactic migration induced by IL-2 or MIP-1 β .* Immune cell migration is the outcome of a subtle biological equilibrium existing between adhesion and detachment events. Lymphocyte adhesion to the subendothelial ECM and subsequent migration are two active processes that can overlap, but are not mutually dependent events. Adhesion and migration may depend on the ability of the
10 T cells to continuously integrate different pro- and anti-adhesive signals via their versatile receptors for ECM, chemokines, cytokines, and possibly, also antigenic moieties. Therefore, we next examined the effects of the Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides (at 1 pg/ml) on the IL-2- and MIP-1 β -induced T cell chemotaxis through FN-coated polycarbonate membranes. The gradient
15 generated by MIP-1 β and IL-2, which were placed in the lower compartment of the 48-well chemotaxis apparatus, induced a marked T cell migration through FN-coated membranes, which was about 3 to 4-fold higher than the control (Fig. 6). Both Ile-Val-Leu, Arg-Met-Leu-Thr (SEQ ID NO:2), and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) markedly ($p<0.01$) inhibited T cell migration toward IL-2, by about 30, 90, and 60%, respectively. However,
20 although the Ile-Val-Leu peptide, and to a lesser degree also the Arg-Met-Leu-Thr (SEQ ID NO:2) peptide, markedly inhibited (80% and 60%, respectively) T cell chemotaxis toward MIP-1 β , the Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) showed only a limited inhibitory effect on the chemokine-induced T cell chemotaxis. Thus, in addition to the capacity of the Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides to inhibit T cell-ECM
25 adhesion, they seem to inhibit T cell migration through FN in response to a diffusible gradient produced by IL-2 or MIP-1 β .

Example 6

Inhibition by the Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides of T cell adhesion to FN induced by various pro-inflammatory mediators and by mAb specific for molecules expressed on T cells. The preceding chemotaxis experiments indicated that the

5 Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptides can inhibit T cell adhesion and migration through FN barriers induced not only by IL-2, but also by the chemokine MIP-1 β . Therefore, in an attempt to further understand the possible physiologic relevance of such phenomena, we examined the ability of these peptides to inhibit the adhesion to FN of T cells stimulated by modes other than IL-2. At 10 pg/ml, both Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) and Ile-Val-Leu inhibited T cell adhesion to FN that was induced by various stimulators of T cells and modulators of the β_1 integrin functions tested (Fig. 7). However, at 0.1 pg/ml, Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) did not inhibit the PMA- and 8A2-induced adhesion, and the Ile-Val-Leu peptide did not inhibit PMA-, 8A2-, and anti-CD3 mAb-induced T cell adhesion to FN, which indicates that these modes of activation are less susceptible to IL-2-derived peptide-induced suppression than IL-2-mediated activation. In experiments similar to those shown in Figs. 7A and 7B, the control peptides (corresponding inverse sequences) did not affect T cell adhesion to FN induced by the indicated activators (data not shown). Hence, Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) and Ile-Val-Leu apparently inhibit T cell adhesion to the FN component of ECM via a common intracellular event that is linked to the regulation of the avidities and affinities of β_1 integrins, and therefore, to their ligand recognition and binding.

Example 7

Inhibition of the reorganization of the actin cytoskeleton in FN-attached activated T cells by the Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptide. The adhesion of immune cells to ECM is dependent on the sequestering of the cytoplasmic domains of integrins in focal adhesion sites, together with actin-containing microfilament bundles (Sanchez-Mateos et al., 1996). Therefore, we examined the effect of Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) on the morphologies of adherent T cells. The T cells were activated with IL-2 or PMA, treated with the IL-2 peptides, and seeded onto FN-coated coverslips. After incubation and

fixation, the actin cytoskeleton of attached T cells was stained with TRITC-conjugated phalloidin. The morphologies of FN-bound IL-2- and PMA-activated T cells (Figs. 8A, 8B) were markedly different from those of non-activated lymphocytes (Fig. 8E); the activated T cells appeared spread, and their actin cytoskeleton performed distinct structures typical of ECM-adherent cells. The Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1) peptide inhibited the redistribution of the actin skeleton in both the IL-2- (Fig. 8C) and PMA-treated (Fig. 8D) FN-adherent T cells. Control peptides (reverse sequences of each peptide **pep1**, **pep2**, **pep3**) did not inhibit the actin reorganization of the activated T cells (not shown). Hence, the adhesion-inhibiting activity of the IL-2-derived peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1), similar to the Ile-Val-Leu peptide (data not shown), appears to involve inhibition of the redistribution of the actin cytoskeleton, and therefore, changes in cell shape and spreading.

Example 8

The effect of synthetic IL-2 derived peptides and derivatives thereof on spontaneous cytokine (IL-8) secretion from intestinal epithelial cells. Human colonic epithelial cell lines, such as HT-29, have been shown to secrete pro-inflammatory cytokines such as IL-1 β and IL-8, in response to cytokines such as TNF- α , IL-1 β , IFN- γ , and to LPS (Ecknamm et al., 1993), or as a response to bacterial invasion (Jung et al., 1995).

The effect of the synthetic IL-2 peptides **pep1**, **pep2**, **pep3**, and other synthetic derivatives of the sequences shown in Table 1 below, was tested on spontaneous IL-8 secretion from HT-29 cells as follows: HT-29 cells (10^6 cells/well) were seeded and incubated in culture medium for 24 h at 37°C in 24-well plates with the desired peptide (1 nM), the supernatants were collected and IL-8 levels were determined by an ELISA assay, using Pharmingen antibody pairs, according to the manufacturer's instructions. The results in Table 1 show the O.D. readings of the ELISA assay (at 405 nm) as well as the percentage of inhibition of IL-8 secretion. A level of inhibition of about 30% or higher indicates that the peptide has relevant anti-inflammatory activity. These results show that the peptides inhibit the spontaneous secretion of IL-8 from a human colonic epithelial cell line and are, therefore, good candidates for the treatment of inflammatory, particularly, bowel diseases.

Table 1: Inhibitory effect of pep1, pep2, pep3 and analogues thereof on spontaneous IL-8 secretion from intestinal epithelial cells

Peptide	IL-8 secretion	
	Average O.D.	% inhibition
Background	71±1.4	
Medium alone	488±8.5	0
(pep1) Ile-Val-Leu,	54±19.1	104
(pep4) Asn-Ile-Asn-Val-Ile-Val-Leu (SEQ ID NO:3),	34±3.5	109
(pep5) Ile-Val-Leu-Glu-Leu-Lys-Gly (SEQ ID NO:4),	35±0.7	109
(pep6) Asn-Val-Ile-Val-Leu (SEQ ID NO:5)	100±14.8	93
(pep7) Ala-Val-Leu	170±8.5	76
(pep8) Ile-Ala-Leu	35±4.9	109
(pep9) Ile-Val-Ala	100±9.9	93
(pep10) Glu-Val-Leu	125±19.1	87
(pep2) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:1)	36±4.2	108
(pep15) Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:8)	33±0.7	109
(pep16) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys (SEQ ID NO:9)	126±7.1	87
(pep17) Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:10)	150±6.4	81
(pep18) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser (SEQ ID NO:11)	162±12.0	78
(pep19) Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:12)	152±30.4	81
(pep20) Arg-Trp-Ile-Thr (SEQ ID NO:13)	191±4.9	71
(pep21) Glu-Phe-Leu-Asn (SEQ ID NO:14)	195±14.1	70

(pep22) Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:15)	158±4.2	79
(pep23) Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:16)	36±14.1	108
(pep24) Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:17)	187±16.3	72
(pep25) Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:18)	172±2.8	76
(pep26) Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr (SEQ ID NO:19)	156±27.6	80
(pep27) Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr (SEQ ID NO:20)	309±18.4	43
(pep28) Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr (SEQ ID NO:21)	120±8.5	88
(pep29) Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr (SEQ ID NO:22)	139±19.8	84
(pep30) Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr (SEQ ID NO:23)	123±9.2	88
(pep31) Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr (SEQ ID NO:24)	112±18.4	90
(pep32) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala (SEQ ID NO:25)	80±24.0	98
(pep33) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-NH ₂ (SEQ ID NO:26)	73±5.7	100
(pep3) Arg-Met-Leu-Thr (SEQ ID NO:2)	44±0.7	107
(pep36) Ala-Met-Leu-Thr (SEQ ID NO:28)	201±17.7	69
(pep37) Arg-Ala-Leu-Thr (SEQ ID NO:29)	222±24.0	64
(pep38) Arg-Met-Ala-Thr (SEQ ID NO:30)	157±1.4	79
(pep39) Arg-Met-Leu-Ala (SEQ ID NO:31)	160±21.9	79
(pep40) Lys-Met-Leu-Thr (SEQ ID NO:32)	179±17.7	74
(pep41) Arg-Val-Leu-Thr (SEQ ID NO:33)	132±17.0	85
(pep42) Arg-Met-Leu-Thr-NH ₂ (SEQ ID NO:34)	226±28.3	63
(pep43) Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr (SEQ ID NO:35)	57±12.7	103
(pep44) Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr (SEQ ID NO:36)	307±29.7	43

The effect of the peptides was also tested on the TNF- α induced secretion of IL-1 β from intestinal epithelial cells. Test cells (HT-29 or Caco-2) were grown as confluent monolayers in 24-well tissue culture plates. After the cells reached confluence, the culture medium was changed and the cells (10⁶ cells/well) were incubated for 24 h at 37°C with the IL-2 peptides (or

control and derivative peptides) in 24-well plates with the addition of TNF- α . The peptides were added to the cells 1 hour prior to the addition of TNF- α . Following culture, the supernatants were harvested and analyzed for IL-1 β secretion by ELISA using a commercially available kit (Genzyme), according to the manufacturers' protocols. The results are similar to those obtained above, the peptides being shown to inhibit TNF- α induced secretion of IL-1 β in similar levels as above (data not shown).

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ANTI-INFLAMMATORY PEPTIDES DERIVED FROM IL-2 AND ANALOGUES**THEREOF****Field of the Invention**

The present invention relates to anti-inflammatory peptides derived from pro-inflammatory IL-2, derivatives thereof, and to pharmaceutical compositions comprising said anti-inflammatory peptides.

Background of the Invention

The migration of T cells through tissues is regulated by adhesion receptors, such as integrins, and by receptors that receive signals provided by pro-inflammatory mediators, such as cytokines, chemokines, and extracellular matrix (ECM)-degrading enzymes (Gilat et al., 1996; Butcher and Picker, 1996).

IL-2 is a 15.5 kDa glycoprotein that participates in the development of inflammation and in the regulation of apoptosis (Taniguchi and Minami, 1993). In addition to its pro-activatory and proliferative roles, IL-2 also induces neutrophil adhesion to umbilical vein endothelial cells in a CD18-mediated manner (Li et al., 1996) as well as chemotactic responses in T cells, both directly and via regulating their expression of CC chemokine receptors (Loetscher et al., 1996). The IL-2 receptor consists of three distinct membrane chains: the α -, β - and γ -chains. Anti-IL-2 antibodies that recognize amino acid epitopes in the N-terminal region of IL-2 can inhibit IL-2-induced lymphocyte proliferation. The C-terminal portion of IL-2 and its three Cys residues seem to contribute to the folding and active conformation of IL-2 (Kuo and Robb, 1986).

The majority of neutrophil elastase (also termed human leukocyte elastase; HLE), which exists as either a membrane-bound or soluble moiety, is produced and released by neutrophils, although small amounts are also produced by macrophages, monocytes, and T cells (Packard et al., 1995). Elastase degrades basement membrane and ECM glycoproteins, such as elastin, collagen, and fibronectin (FN), as well as molecules expressed on the surface of T cells, e.g., CD4, CD8, and CD2 (Doring et al., 1995).

Summary of the Invention

It has now been surprisingly observed, according to the present invention, that enzymatic degradation of IL-2 by proteolytic enzymes that participate in the breakdown of the extracellular matrix (ECM) results in small peptidic fragments that possess anti-inflammatory activity.

A first aspect of the present invention therefore relates to anti-inflammatory peptides derived from IL-2, and to anti-inflammatory derivatives of said peptides.

The anti-inflammatory peptides of the present invention are defined as such by virtue of their ability to inhibit at least one of the following processes in vitro: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells.

A second aspect of the present invention relates to a method for identifying anti-inflammatory peptides derived from IL-2, which comprises:

(i) carrying out enzymatic digestion of IL-2 with a proteolytic enzyme that participates in the breakdown of the extracellular matrix (ECM);

(ii) testing the fractions obtained in (i) for their in vitro ability to inhibit at least one of the following processes: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells;

(iii) selecting the fractions of (ii) active in at least one of the bioassays (a) to (e), fractionating each fraction to isolate individual peptides thereof, and submitting each isolated peptide to sequencing and synthesis; and

(iv) carrying out one or more of the bioassays (a) to (e) with a peptide isolated in step (iii), and selecting those peptides that exhibit anti-inflammatory activity in at least one of said

bioassays.

The second aspect of the invention further relates to the peptides obtainable from the method of identification described above and peptide derivatives thereof.

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The proteolytic enzyme is one capable of processing the glycoprotein constituents of tissues and blood vessel walls and may be elastase, a collagenase or a metalloprotease such as metalloelastase, matrix metalloprotease-2 (MMP-2) and matrix metalloprotease-9 (MMP-9). In a preferred embodiment, the proteolytic enzyme is elastase.

10

The anti-inflammatory synthetic peptides of the present invention have been characterized by their possession of inhibitory activity in at least one of processes (a) to (e) given above, the anti-inflammatory peptide being preferably active in at least one of assays (a), (b) or (e).

15

A third aspect of the invention relates to pharmaceutical compositions comprising at least one anti-inflammatory synthetic peptide of the invention and a pharmaceutically acceptable carrier. This aspect includes also the use of said peptides for the preparation of pharmaceutical compositions for the treatment and/or alleviation of acute and chronic inflammatory disorders.

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A fourth aspect of the invention relates to a method for the treatment and/or alleviation of acute and chronic inflammatory disorders comprising administering to a subject in need thereof an effective amount of an anti-inflammatory synthetic peptide of the invention.

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Brief Description of the Drawings

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Figs. 1A-1B show induction of T cell adhesion to fibronectin (FN), laminin (LN), collagen type IV (CO-IV) (Fig. 1A) and ECM (Fig. 1B) by IL-2. [^{51}Cr]-Labeled human T cells were seeded onto FN-, LN-, CO-IV-, and ECM-coated microtiter wells together with IL-2. After 30 min at 37°C, non-adherent T cells were removed, the adherent cells were lysed, and the percentage of T cells that had adhered was determined. One experiment representative of four.

Fig. 2 depicts a chromatogram of IL-2 fractions obtained upon elastase proteolysis. IL-2 (1mg/ml) was incubated (12 hr, 37°C) with neutrophil elastase (50µg/ml in PBS). The resulting enzymatic digests were purified by HPLC. Fraction 1 consisted of salts used for the separation procedure. One experiment representative of three.

5

Fig. 3 shows effects of IL-2 fractions, generated by elastase degradation, on the IL-2-induced adhesion of T cells to FN. IL-2 (10 U/ml)-stimulated [⁵¹Cr]-labeled T cells were seeded, in the presence or absence of elastase-generated IL-2 protein products, onto wells coated with FN. After 30 min at 37°C, non-adherent T cells were removed, and the percentage of adhered cells was determined. One experiment representative of six.

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Figs. 4A-4C show specific inhibition, by elastase-generated fractions 2 and 8 of IL-2 and by their synthetic peptides, of IL-2-induced T cell adhesion to FN. Fig. 4A: T cell adhesion to FN in the presence of IL-2 fractions and the IL-2 peptides Ile-Val-Leu (IVL), Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (EFLNRWIT), and Arg-Met-Leu-Thr (RMLT), herein designated **pep1**, **pep2** and **pep3**, respectively. Fig. 4B. The effects on T cell adhesion to FN of the inversely synthesized IL-2 peptides Leu-Val-Ile (LVI), Thr-Leu-Met-Arg (TLMR) and Thr-Ile-Trp-Arg-Asn-Leu-Phe-Glu (TIWRNLFE). T cells were activated with IL-2 (10 U/ml) and seeded onto FN-coated wells in the presence of IL-2, fraction 2, fraction 8, or inversed IL-2 peptides. Fig. 4C. Effects of pre-treatments of T cells with the IL-2 peptides **pep1**, **pep2** and **pep3**, on the subsequent IL-2-induced T cell adhesion to FN. T cells used were untreated or pre-treated with the indicated peptides (1 pg/ml; 60 min, 37°C, 10% CO₂, humidified atmosphere), washed twice, exposed to IL-2, and seeded onto the FN-coated wells. After 30 min at 37°C, T cell adhesion was measured. One experiment representative of five.

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Fig. 5 shows inhibition by the IL-2 peptides **pep1**, **pep2** and **pep3**, of T cell adhesion to LN, CO-IV and FN. T cells were pre-treated with the indicated IL-2 peptides (10 pg/ml, 30 min, 37°C, 10% CO₂, humidified atmosphere) and then with IL-2 (10 U/ml). The T cells were then seeded onto microtiter wells that were pre-coated (1 µg/well) with the various ECM glycoproteins. T cell adhesion was measured 30 min later.

30

Fig. 6 shows inhibition of IL-2-induced T cell migration through FN by **pep1** and **pep3**. T

cells were pre-treated with the IL-2 peptides (1 pg/ml) or buffer alone and then placed in the upper wells of a chemotaxis chamber, in which IL-2 (10 U/ml) or MIP-1 β (10 ng/ml) had been added to the lower compartment. T cell migration towards the chemotactic sources was assessed after 2 hr. One experiment representative of four.

5 Figs. 7A-7B show inhibition by **pep2** (Fig. 7A) and **pep1** (Fig. 7B) of T cell adhesion to FN, induced by various activators. Labeled T cells were seeded onto FN-coated wells in the presence of IL-2 (10 U/ml), IL-7 (50 ng/ml), MIP-1 β (20 ng/ml), PMA (50 ng/ml), 8A2 mAb (1 μ g/ml), or anti-human CD3 mAb (1 μ g/ml). The IL-2-derived peptides, **pep1** or **pep2**, were
10 also present in some wells. After 30 min at 37°C, non-adherent T cells were removed by washing, the remaining adherent cells were lysed, and the percentage of T cells that had adhered was determined. One experiment representative of four.

15 Figs. 8A-8E show inhibition by **pep2** of the spreading and redistribution of the actin cytoskeleton in IL-2- and PMA-activated FN-adherent T cells. T cells were activated (48 hr) with IL-2 (50 U/ml). The T cells were then washed and seeded onto FN-coated coverslips, in medium alone (8E), or in the presence of IL-2 (100 U/ml; 8A and 8C), PMA (50 ng/ml; 8B and 8D), or **pep2** (10 pg/ml; 8C and 8D). After incubation, the intracellular actin filaments of the fixed FN-attached T cells were stained. Original magnification, x1000.

20 Detailed Description of the Invention

IL-2 and elastase are prominent mediators of leukocyte extravasation and migration from the vasculature through the ECM to sites of inflammation. The present invention is based on the assumption that, in contrast to the pro-adhesive effects of the intact IL-2 molecule, certain
25 short IL-2-derived peptides, which may occur in vivo, can inhibit the interactions of T cells with ECM, and that this interference is independent of the effects of the peptides on the activation of T cells by IL-2. We also assumed that such moieties of IL-2 can prevent the arrival of T cells at inflamed sites.

30 An important feature of elastase is its ability to act in both soluble and immobilized forms, since a migrating immune cell that expresses immobilized elastase may encounter matrix-bound IL-2, among other cytokines. This made neutrophil elastase a likely candidate

for the physiologic production of these inhibitory peptides. Indeed, it was found, according to the present invention, that the processing of recombinant IL-2 by elastase resulted in the production of at least eight different by-products.

- 5 Three of the products generated by processing of recombinant IL-2 by neutrophil elastase (present in HPLC fractions 2, 7 and 8), inhibited IL-2-mediated T cell adhesion to FN. Amino acid composition analysis and amino acid sequencing revealed that fraction 2 contained the tripeptide Ile-Val-Leu (IL-2₁₁₂₋₁₁₄) (herein designated **pep1**) and the tetrapeptide Arg-Met-Leu-Thr (IL-2₅₈₋₆₁) (herein designated **pep3**), and fraction 8 the octapeptide
- 10 Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (IL-2₁₃₆₋₁₄₃) (herein designated **pep2**). The **pep3** appeared to be located within the IL-2-binding site of the α -chain of the IL-2R, whereas **pep1** and **pep2** are located at sites far from the receptor-binding sites of IL-2. These peptides, at a picomolar range of concentrations (i.e., 0.01-1 pg/ml), inhibited the IL-2, as well as MIP-1 β -induced chemotaxis of human T cells through FN-coated polycarbonate membranes.

15 These peptides were synthesized and the synthetic peptides were shown to exhibit the same activity as the IL-2 digested fractions 2 and 8. Thus, in one preferred embodiment, the IL-2 derived peptides of the invention are the peptides:

- (**pep1**) Ile-Val-Leu,
- 20 (**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr
- (**pep3**) Arg-Met-Leu-Thr,

- 25 The chemoattractive capacity of IL-2 in T cell migration studies in vitro has been shown by using bare polycarbonate filters or collagen- or Matrigel-coated membranes as immobilized substrates. T cell migration in these systems was proved to be IL-2R β -chain-specific and depended on the activities of the matrix-degrading gelatinases (Leppert et al., 1995). According to the present invention, the IL-2 peptides **pep1**, **pep2**, **pep3**, in addition to their anti-migratory effects, also inhibited T cell adhesion to FN induced by various physiologic and non-physiologic stimuli. Nevertheless, none of them, at 1 to 100 pg/ml, interfered with
- 30 either PHA- or IL-2-mediated proliferative responses of human T cells, nor did these peptides inhibit the secretion of TNF- α and IFN- γ from these proliferating cells (not shown).

Moreover, the inversely synthesized molecules, Leu-Val-Ile, Thr-Leu-Met-Arg, and Thr-Ile-Trp-Arg-Asn-Leu-Phe-Glu, did not inhibit T cell adhesion to FN. Thus, the migration- and adhesion-suppressive capabilities of **pep1**, **pep2** and **pep3** are specific, and are not due to toxic cell death.

5

How do the elastase-derived IL-2 peptides exert their inhibitory functions? We have demonstrated, according to the present invention, that the three peptides **pep1**, **pep2** and **pep3** do not have to be present during the entire period of the adhesion assay, since their anti-adhesive effect was apparent even after their removal from the assay prior to T cell activation with IL-2. This finding also implies that **pep1**, **pep2** and **pep3** do not function by interacting with putative cell-adhesive epitopes present on the tested ECM glycoproteins. It seems to be highly unlikely that two of these peptides (**pep1** and **pep2**) exert their biologic functions by interacting with the IL-2R subunits or by directly binding to β_1 -specific integrins. The IL-2 peptides interfered with different modes of T cell activation, leading to their adhesion to the tested matrix proteins. Moreover, both **pep1** and **pep2**, used in a picomolar range of concentrations, appear to block T cell adhesion not only to FN, mediated predominantly via the $\alpha_4\beta_1$ and $\alpha_5\beta_1$ integrins, but also to LN; T cell adhesion to LN was mediated primarily through the $\alpha_3\beta_1$ and $\alpha_6\beta_1$ integrins. Interesting, however, is the fact that **pep3** (Arg-Met-Leu-Thr) resides within the IL-2R α -binding site of IL-2; two residues, Arg⁵⁸ and Phe⁶², which are present within and adjacent to this peptide, respectively, were shown to be critical for IL-2-IL-2R interactions. Therefore, it will also be interesting to examine whether the degradation of IL-2 by elastase produces compounds that can interfere with IL-2 binding to its receptor and with the biologic outcome of such molecular interactions.

The ability of **pep2** as well as **pep1** and **pep3** (data not shown) to inhibit the PMA- and IL-2-induced T cell adhesion to FN is probably linked to its ability to block the reorganization of the intracellular actin cytoskeleton. Integrin-cytoskeleton associations can modulate cell adhesion to ECM ligands, cell spreading in areas of cell contact with the substratum, and the micro-clustering and redistribution of β_1 integrins on the cell surface at sites of focal adhesion located at the ends of the actin fibers (Otey et al., 1990). Similar observations were noted for T cell activation by IL-2 (and PMA) and attachment to FN. Although the intracellular

mechanisms of action of the elastase-generated fractions and peptides have not yet been determined, we postulate that these proteins effect the adhesion and migration of T cells within the ECM by active inhibition of intracellular signal transduction pathways linked to cytoskeleton organization, resulting in an inhibition of micro-clustering and an association of integrins with cytoskeletal elements.

Our findings according to the present invention imply that the tissue-invading T cells themselves can dynamically regulate their own functions. Both adhesion- and migration-promoting stimuli (i.e. intact IL-2) and suppressive by-products of inflammatory mediators can be present, although not necessarily simultaneously, within the inflammatory milieu. At the early stages of inflammation, both IL-2 and elastase may function concomitantly to activate T cells to penetrate tissues. Later, the degradation peptide products of IL-2, generated by elastase, may inhibit T cell migration, inhibit the co-stimulatory effects of IL-2 and other mediators, and probably signal the termination of the inflammatory reaction.

As stated before, the invention relates to anti-inflammatory peptides derived from the IL-2 sequence and to derivatives thereof, said anti-inflammatory activity being assessed by their ability to inhibit at least one of the following processes in vitro: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine such as IL-1 β or, preferably, IL-8, from intestinal epithelial cells.

The in vitro bioassays (a) to (e) are well-known in the art. Thus, for example, assays (a), (b) and (e) may be carried out as described in the section Materials and Methods hereinafter. Assay (c), for inhibition of cytokine - or mitogen-induced T cell proliferation, is carried out as follows: A total of 25×10^5 of human T cells in 0.2 ml of RPMI medium supplemented with 5% heat-inactivated FCS (Gibco), antibiotics, 1 % Hepes buffer, 1% glutamine and 5×10^{-5} M 2-mercaptoethanol (ME), are added per well. T cell activation is tested by adding either the cytokine IL-2 (100 U/ml) or the mitogen Con-A (2.5 μ g/ml) to the culture wells in the beginning of the assay. The IL-2-derived peptides or their derivatives are added to the culture wells (different amounts, 10 microliters) for the entire period of the assay. [3 H]-Thymidine is

added to the microtiter 96 U-form well plates 18h before harvesting the cultures on day 3 onto glass fiber filters and counted by liquid scintillation counter. Mean CPM and SD from quadruplicate culture are then presented. Assay (d), for inhibition of cytokine secretion by cytokine- or mitogen-stimulated T cells, is carried out similar to the proliferation assay (c), except for one modification: the supernatants of activated T cells (or any other leukocytes derived from human blood), treated with the different peptides, are collected and assayed for IL-2 and TNF- α , using conventional ELISA kits.

In vivo bioassays may also be used to determine anti-inflammatory activity of the peptides of the invention, examples of which include: (1) inhibition of experimental delayed type hypersensitivity (DTH) reactivity carried out, for example, as described in PCT Publication No. WO 94/11006, Example 5.2; (2) treatment of experimental adjuvant arthritis (AA) in rats carried out, for example, as described in PCT Publication No. WO 94/11006, Example 5.7; and (3) treatment of experimental autoimmune encephalomyelitis (EAE) in guinea pigs, a model disease for multiple sclerosis, carried out, for example, as described in US Patent No. 5,206,223.

The term "peptides of the invention" as used hereinafter includes the synthetic peptides **pep1**, **pep2**, and **pep3** derived from IL-2 as well as synthetic analogues thereof, and comprise:

(i) peptides **pep1**, **pep2**, and **pep3** of the sequences:

(**pep1**) Ile-Val-Leu

(**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr

(**pep3**) Arg-Met-Leu-Thr

(ii) peptides obtained from **pep2** by deletion of one or more amino acid residues;

(iii) peptides obtained by addition to peptides (i) or (ii) of one or more natural or non-natural amino acid residues;

(iv) peptides obtained by replacement of one or more amino acid residues of peptides (i) to (iii) by the corresponding D-stereomer, by another natural amino acid residue or by a non-natural amino acid residue;

(v) chemical derivatives of the peptides (i) to (iv);

(vi) cyclic derivatives of peptides (i) to (v);

(vii) dual peptides consisting of two of the same or different peptides (i) to (vi),

wherein the peptides are covalently linked to one another directly or through a spacer; and
 (viii) multimers comprising a number of the same or different peptides (i) to (vi).

The term "peptide derivative" or "peptide analogue" as used throughout the specification and
 5 claims herein is intended to include the derivatives defined in (ii) to (viii) above, namely
 peptides obtained by deletion, addition, or replacement of a natural amino acid residue by the
 corresponding D-stereomer or by a non-natural amino acid residue, chemical derivatives of
 the peptides, cyclic peptides, dual peptides and multimers of the peptides.

10 Typically, modifications are made that retain the anti-inflammatory effect of the parent
 peptides **pep1**, **pep2** and **pep3**. Any of the above modifications may be utilized alone or in
 combination, provided that the modified sequence retains anti-inflammatory activity,
 preferably at least 30% of the anti-inflammatory activity, more preferably at least the same
 activity or higher, of the parent peptide, identified by means of the above described assays.

15 Deletion of 1-4 amino acid residues of **pep2** may be made at the N- or the C-terminal such as
 for example peptides **pep19** and **pep21**, respectively, in Table 1 hereinafter. Addition of one
 or more natural or non-natural amino acid residues may be made at the N- or the C-terminal
 of each of the parent peptides **pep1**, **pep2** and **pep3**, such as for example peptides **pep4**, **pep5**
 20 and **pep6** (analogues of **pep1**), the peptides **pep15** and **pep17** (analogues of **pep2**), and the
 peptide **pep43** (analogue of **pep3**).

Substitutions include replacement of the natural amino acid residues by the corresponding
 D-amino acid residue, for example to increase blood plasma half-life of a therapeutically
 25 administered peptide, or by different natural amino acid residues or by non-natural amino acid
 residues. Thus, the peptide or peptide derivative of the invention may be all-L, all-D or a
 D,L-peptide. Examples of non-natural amino acids include, but are not limited to, N α -alkyl,
 particularly N α -methyl, amino acids*, C α -alkyl, particularly C α -methyl, amino acids*, halo
 derivatives of natural amino acids such as trifluorotyrosine*, p-Cl-phenylalanine*,
 30 p-Br-phenylalanine* and p-I-phenylalanine*, β -alanine (β -Ala)*, L- α -aminobutyric acid*,
 L- γ -aminobutyric acid (γ -Abu)*, L- α -aminoisobutyric acid (α -Aib)*, L- ϵ -aminocaproic acid*,
 7-aminoheptanoic acid*, L-norleucine (Nle)*, L-norvaline (Nva)*, L-p-nitro-phenylalanine*,

L-hydroxyproline (Hyp)*, L-thioprolin*, ornithine (Orn)**, homoarginine (homoArg)#, diaminobutyric acid (Dab)**, pyridylalanine*, thienylalanine*, naphthylalanine*, phenylglycine*, methyl derivatives of phenylalanine (Phe) such as L-4-methyl-Phe* and L-pentamethyl-Phe*, L-4-amino-Phe*, L-4-isopropyl-Phe*, L-4-phenyl-Phe*,
 5 L-4-benzyl-Phe*, 1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (L-Tic)*, L-methyl-Tyr*, and L-diaminopropionic acid (Dap) **. The notation * has been utilised to indicate the hydrophobic nature of the moiety whereas # has been utilised to indicate the hydrophilic nature of the derivative, and ** indicates amphipathic characteristics.

10 A "chemical derivative" of a peptide of the invention includes, but is not limited to, a derivative containing additional chemical moieties not normally a part of the peptide provided that the derivative retains the anti-inflammatory function of the peptide. Examples of such derivatives are: (a) N-acyl derivatives of the amino terminal or of another free amino group, wherein the acyl group may be either an alkanoyl group, e.g. acetyl, hexanoyl, octanoyl, or an
 15 aroyl group, e.g. benzoyl; (b) esters of the carboxy terminal or of another free carboxy or hydroxy groups; (c) amides of the carboxy terminal or of another free carboxy groups produced by reaction with ammonia or with a suitable amine, resulting in the C-terminus or another carboxy group being in the form -C(O)-NH-R, wherein R may be hydrogen, C1-6 alkyl, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, pentyl or hexyl, aryl such as
 20 phenyl, and aralkyl such as benzyl, such amidation being advantageous in providing additional stability and possibly enhanced activity to the peptide; (d) glycosylated derivatives; (e) phosphorylated derivatives; (f) derivatives conjugated to lipophilic moieties e.g. caproyl, lauryl, stearoyl; and (g) derivatives conjugated to an antibody or other cellular ligands. Also included among the chemical derivatives are those derivatives obtained by modification of
 25 the peptide bond -CO-NH-, for example by (a) reduction to -CH₂-NH- ; (b) alkylation to -CO-N (alkyl)- ; (c) inversion to -NH-CO- .

The term "cyclic peptides" as used herein are cyclic derivatives containing either an intramolecular disulfide bond, i.e. -S-S-, an intramolecular amide bond, i.e. -CONH- or
 30 -NHCO-, or intramolecular S-alkyl bonds, i.e. -S-(CH₂)_n-CONH- or -NH-CO(CH₂)_n-S-, wherein n is 1 or 2. The cyclic derivatives containing an intramolecular disulfide bond may be prepared by conventional solid phase synthesis while incorporating suitable S-protected

cysteine or homocysteine residues at the positions selected for cyclization such as the amino and carboxy terminals of the peptides, with the option of including spacing residues, such as (Ala)_n, (Gly)_n where n is from 1 to 4, or non-natural amino acids such as 6-aminocaproic acid, between the terminal residue and the linking residue. The linking residues may then be
5 linked together using known techniques to form cyclicized peptide derivatives. For example, the linear peptides Cys-Ile-Val-Leu-Ala-Cys, Cys-Ile-Val-Leu-Ala-Ala-Cys, Cys-Arg-Met-Leu-Thr-Ala-Cys and Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys were prepared and cyclized according to methods known in the art for formation of a disulphide bond. Following completion of the chain assembly, cyclization can be performed either by
10 selective removal of the S-protecting groups with a consequent on-support oxidation of free corresponding two SH-functions, to form S-S bonds, followed by conventional removal of the product from the support and appropriate purification procedure, or by removal of the peptide from the support along with complete side-chain deprotection, followed by oxidation of the free SH-functions in highly dilute aqueous solution. The cyclic derivatives containing an
15 intramolecular amide bond may be prepared by conventional solid phase synthesis while incorporating suitable amino and carboxyl side-chain protected amino acid derivatives at the positions selected for cyclization. The cyclic derivatives containing intramolecular -S-alkyl bonds may be prepared by conventional solid phase synthesis while incorporating an amino acid residue with a suitable amino-protected side chain, and a suitable S-protected cysteine or
20 homocysteine residue at the positions selected for cyclization.

A "dual peptide" according to the invention consists of two the same or different peptides or peptide derivatives of the invention covalently linked to one another directly or through a spacer such as by a short stretch of alanine residues or by a putative site for proteolysis by
25 cathepsin (see U.S. Patent No. 5,126,249 and European Patent No. 495,049 with respect to such sites). This will induce site-specific proteolysis of the preferred form into the two desired analogues.

"Multimers" according to the invention consist of polymer molecules formed from a number
30 of the same or different peptides or derivatives thereof. The polymerization is carried out with a suitable polymerization agent, such as 0.1% glutaraldehyde (Audibert et al. (1981) Nature, 289 : 593)

Modifications to the amino acid residues in the peptides of the present invention are discussed below using nomenclature as given in the Table below:

ALIPHATIC	Non-polar, neutral	Gly Ala Pro
low hydrophobic		
high hydrophobic		Ile Leu Val Met
low hydrophilic	Polar, neutral	Cys Ser Thr
high hydrophilic		Asn Gln
Hydrophilic	Polar – negatively charged	Asp Glu
	Polar – positively charged	Lys Arg His
AROMATIC hydrophobic		Phe Trp Tyr

Thus, in one preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Ile-Val-Leu (**pep1**), and to anti-inflammatory peptides resulting from the modification thereof by:

- (a) elongation by up to 3-4 further amino acid residues at the N- and/or C-terminal, such as in **pep11** and **pep13**, or preferably according to the natural sequence of IL-2;
- (b) substitution of the Ile residue by a natural or non-natural amino acid hydrophilic polar neutral or negatively charged, or hydrophobic non-polar neutral amino acid residue, preferably selected from Glu, Asp, Asn, Gln, Ala, Val;
- (c) substitution of the Val residue by a hydrophobic, non-charged natural or non-natural amino acid residue, preferably selected from Ala, Ile, Leu, Met, Nle, Phe;
- (d) substitution of the Leu residue by a hydrophobic, non-charged natural or non-natural amino acid residue, preferably selected from Ala, Ile, Met, Nle, Phe, Val;
- (e) amidation of the C-terminal Leu residue,
- (f) cyclization of **pep1** or of any peptide of (a) to (e); and
- (g) any combination of (a) to (f).

Examples of such modified peptides derived from **pep1** include;

(**pep4**) Asn-Ile-Asn-Val-Ile-Val-Leu,

(**pep5**) Ile-Val-Leu-Glu-Leu-Lys-Gly,

5 (**pep6**) Asn-Val-Ile-Val-Leu

(**pep7**) Ala-Val-Leu

(**pep8**) Ile-Ala-Leu

(**pep9**) Ile-Val-Ala

(**pep10**) Glu-Val-Leu

10 (**pep11**, linear) and (**pep12**, cyclic) Cys-Ile-Val-Leu-Ala-Cys and,

(**pep13**, linear) and (**pep14**, cyclic) Cys-Ile-Val-Leu-Ala-Ala-Cys

In a further preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (**pep2**), and to anti-inflammatory peptides resulting from the modification thereof by;

(a) elongation by up to 4 further amino acid residues at the C and/or N terminal ends, such as in **pep34**, or preferably according to the natural sequence of IL-2;

(b) substitution of the Glu residue by a natural or non-natural charged or polar charged amino acid residue, preferably selected from Lys, Arg, Asp, Gln, Asn;

20 (c) substitution of the Phe residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(d) substitution of the Leu residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

25 (e) substitution of the important Asn residue by a hydrophilic, non-charged, aliphatic natural or non-natural amino acid residue such as Gln;

(f) substitution of the Arg residue by a positively charged, natural or non-natural amino acid residue, preferably selected from Lys, Orn, homoArg;

30 (g) substitution of the Trp residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Ile, Leu, Nle, Tic, Phe, 4-phenyl-Phe, 4-methyl-Phe;

- (h) substitution of the Ile residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Phe, Leu, Nle, Tic;
- (i) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue preferably selected from Cys, Ser;
- (j) truncation by up to 4 amino acid residues from either the C or N terminal;
- (k) amidation of the C-terminal Thr;
- (l) cyclization of **pep2** or of any peptide of (a) to (k); and
- (m) any combination of (a) to (l).

Examples of such modified peptides derived from **pep2** include:

- | | |
|---|--|
| (pep15) | Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr |
| (pep16) | Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys |
| (pep17) | Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr |
| (pep18) | Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser |
| (pep19) | Leu-Asn-Arg-Trp-Ile-Thr |
| (pep20) | Arg-Trp-Ile-Thr |
| (pep21) | Glu-Phe-Leu-Asn |
| (pep22) | Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr |
| (pep23) | Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr |
| (pep24) | Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr |
| (pep25) | Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr |
| (pep26) | Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr |
| (pep27) | Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr |
| (pep28) | Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr |
| (pep29) | Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr |
| (pep30) | Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr |
| (pep31) | Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr |
| (pep32) | Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala |
| (pep33) | Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-NH ₂ and, |
| (pep34 , linear) and (pep35 , cyclic) | Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys. |

In a further preferred embodiment, the present invention relates to the synthetic anti-inflammatory peptide Arg-Met-Leu-Thr (**pep3**), and to anti-inflammatory peptides resulting from the modification thereof by:

- (a) elongation by up to 4 further amino acid residues at the C and/or N terminal end, such as in **pep45**, or preferably according to the natural sequence of IL-2;
- (b) substitution of the Arg residue by a natural or non-natural positively charged amino acid residue, preferably selected from Lys, Orn, homoArg, diaminobutyric acid;
- (c) substitution of the Met residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Ile, Leu, Nle, Tic;
- (d) substitution of the Leu residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Nle, Tic;
- (e) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue such as Ser, Cys;
- (f) amidation of the C-terminal Thr residue;
- (g) cyclization of **pep3** or of any peptide of (a) to (f); and
- (h) any combination of (a) to (g).

Examples of such modified peptides derived from **pep3** include;

- (**pep36**) Ala-Met-Leu-Thr
- (**pep37**) Arg-Ala-Leu-Thr
- (**pep38**) Arg-Met-Ala-Thr
- (**pep39**) Arg-Met-Leu-Ala
- (**pep40**) Lys-Met-Leu-Thr
- (**pep41**) Arg-Val-Leu-Thr
- (**pep42**) Arg-Met-Leu-Thr-NH₂
- (**pep43**) Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr
- (**pep44**) Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr and,
- (**pep45**, linear) and (**pep46**, cyclic) Cys-Arg-Met-Leu-Thr-Ala-Cys.

The peptides and peptide derivatives of the invention are obtained by any method of peptide synthesis known to those skilled in the art, such as for example by solid phase peptide

synthesis.

The present invention is also directed to pharmaceutical compositions comprising a pharmaceutically acceptable carrier and at least one anti-inflammatory peptide or peptide derivative of the invention. The pharmaceutical composition will be administered according to known modes of peptide administration, including oral, intravenous, subcutaneous, intraarticular, intramuscular, inhalation, intranasal, intrathecal, intradermal, transdermal or other known routes. The dosage administered will be dependent upon the age, sex, health condition and weight of the recipient, and the nature of the effect desired.

The peptides of the invention for use in therapy are typically formulated for administration to patients with a pharmaceutically acceptable carrier or diluent to produce a pharmaceutical composition. The formulation will depend upon the nature of the peptide and the route of administration but typically they can be formulated for topical, parenteral, intramuscular, intravenous, intraperitoneal, intranasal inhalation, lung inhalation, intradermal or intra-articular administration. The peptide may be used in an injectable form. It may therefore be mixed with any vehicle which is pharmaceutically acceptable for an injectable formulation, preferably for a direct injection at the site to be treated, although it may be administered systemically.

The pharmaceutically acceptable carrier or diluent may be, for example, sterile isotonic saline solutions, or other isotonic solutions such as phosphate-buffered saline. The peptides of the present invention may be admixed with any suitable binder(s), lubricant(s), suspending agent(s), coating agent(s), solubilising agent(s). It is also preferred to formulate the peptide in an orally active form.

Tablets or capsules of the peptides may be administered singly or two or more at a time, as appropriate. It is also possible to administer the peptides in sustained release formulations.

Typically, the physician will determine the actual dosage which will be most suitable for an individual patient and it will vary with the age, weight and response of the particular patient. There can, of course, be individual instances where higher or lower dosage ranges are merited, and such are within the scope of this invention.

Alternatively, the peptides of the invention, can be administered by inhalation or in the form of a suppository or pessary, or they may be applied topically in the form of a lotion, solution, cream, ointment or dusting powder. An alternative means of transdermal administration is by use of a skin patch. For example, they can be incorporated into a cream consisting of an aqueous emulsion of polyethylene glycols or liquid paraffin. They can also be incorporated, at a concentration of between 1 and 10% by weight, into an ointment consisting of a white wax or white soft paraffin base together with such stabilisers and preservatives as may be required.

For some applications, preferably the compositions are administered orally in the form of tablets containing excipients such as starch or lactose, or in capsules or ovules either alone or in admixture with excipients, or in the form of elixirs, solutions or suspensions containing flavouring or colouring agents. For such oral administration, the peptide may preferably formed into microcapsules or nanoparticles together with biocompatible polymers such as poly-lactic acid and the like.

The compositions (as well as the peptides alone) can also be injected parenterally, for example intracavernosally, intravenously, intramuscularly or subcutaneously. In this case, the compositions will comprise a suitable carrier or diluent. For parenteral administration, the compositions are best used in the form of a sterile aqueous solution which may contain other substances, for example enough salts or monosaccharides to make the solution isotonic with blood.

For buccal or sublingual administration the compositions may be administered in the form of tablets or lozenges which can be formulated in a conventional manner.

The peptides and peptide derivatives of the invention are for use in the treatment of or amelioration of acute and chronic inflammatory disorders including, but not being limited to, autoimmune diseases such as rheumatoid arthritis, diabetes type I, multiple sclerosis, systemic lupus erythematosus, uveitis, bowel inflammation and Crohn's disease.

The present invention further relates to a method of treatment of a patient suffering from an inflammatory disorder which comprises administering to said patient an effective amount of a peptide or peptide derivative of the invention.

- 5 The invention will now be illustrated by the following non-limiting Examples.

EXAMPLES

Material and methods

- 10 *Materials.* The following reagents were obtained as indicated. Recombinant human IL-2 (sp. act. 18×10^6 U/mg; Chiron B.V., Amsterdam, The Netherlands); recombinant human IL-7 (sp. act. 2×10^5 U/mg; Immunex Corp., Seattle, WA); recombinant human macrophage inflammatory protein MIP-1 β (Pepro Tech, Rocky Hill, NJ); recombinant human TNF- α (Boehringer, Ingelheim, Germany); Affi-gel 10 beads (Pharmacia, NJ); fibronectin (FN) (Chemicon; Temecula, CA); BSA, laminin (LN), PMA, and TRITC-conjugated phalloidin (Sigma
15 Chemical Co., St. Louis, MO); collagen type IV (CO-IV; ICN, Costa Mesa, CA); and HEPES buffer, antibiotics, heat-inactivated FCS, sodium pyruvate, DMEM, CSS and RPMI-1640 (Beit-Haemek, Israel). An anti- β_1 integrin-specific affinity-modulating mAb, 8A2, was donated by Dr. J. M. Harlan (Washington University, Seattle, WA). All protected amino
20 acids, coupling reagents, and polymers were obtained from Nova Biochemicals (Läufelfingen, Switzerland). Synthesis-grade solvents were obtained from Labscan (Dublin, Ireland). HPLC solvents and columns were obtained from Merck (Darmstadt, Germany).

- Human colonic epithelial cell lines HT-29 (ATCC HTB38) and Caco-2 (ATCC HTB27)
25 were obtained from the American Type Culture Collection (ATCC, Rockville, MD). Cells were maintained in culture medium (DMEM supplemented with 10% cosmic calf serum (CSS), 2 mM glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin), at 37°C and atmosphere of 5% CO₂. Cell viability was assessed using the standard MTT method.

- 30 *T cell adhesion assays.* Human T cells were purified from the peripheral blood of healthy donors, and T cell adhesion to immobilized protein substrates was examined as follows: Human leukocytes were isolated on a Ficoll gradient, washed, and incubated (2 h, 37°C, 7.5%

CO₂, humidified atmosphere) on petri dishes. The non-adherent cells were then collected and incubated (1 h, 37°C, 7.5% CO₂, humidified atmosphere) on nylon wool columns (Novamed Ltd., Jerusalem, Israel). Unbound cells were eluted from the columns by extensive washings. The resulting cell population was always >92% T cells. Intact IL-2 or IL-2-derived peptides (in 50 µl) were added to flat-bottom microtiter wells that had been pre-coated with ECM or ECM proteins (FN or LN; 1 µg/well) and blocked with 0.1% BSA. After 4-6 h at 37°C, the wells were washed and ⁵¹[Cr]-labeled T cells were added to the wells, 10⁵ cells/100 µl of adhesion medium (RPMI-1640 supplemented with 0.1% BSA, 1% sodium pyruvate, 1% HEPES buffer). The microtiter plates containing the cells were incubated (30 min, 37°C) in a humidified, 7.5% CO₂ atmosphere, and then washed. The adherent cells were lysed, and the resulting supernatants were removed and analyzed in a γ-counter. For each experimental group, the results were expressed as the mean percentage ±SD of bound T cells from quadruplicate wells. To some wells, different concentrations of soluble IL-2 were added concomitantly with the T cells, and with others, different concentrations of elastase-degraded IL-2-derived fractions, or the corresponding synthetic peptides, were added together with stimulators [PMA (50 ng/ml), IL-7 (50 ng/ml), MIP-1β (20 ng/ml), 8A2 (1 µg/ml), mAb anti-CD3 (1 µg/ml), or IL-2 (10 U/ml)].

Chemotaxis assays. T cell chemotaxis was performed and analyzed as previously described (Ben-Baruch et al., 1997). Briefly, the migration of human T cells (0.5x10⁶ cells in adhesion medium/well) was examined in a 48-well chemotaxis micro-chamber (Neuro-Probe Inc., Cabin John, MD). The two compartments of the micro-chambers were separated by a FN-coated polycarbonate filter (5-µm pore size; Osmonics Protein Products, Livemore, CA). Where indicated, MIP-1β or IL-2 was added to the lower wells, and the T cells were added to the upper chambers together with the peptides. After incubation (120 min, 37°C, in a humidified, 7.5% CO₂ atmosphere), the filters were removed, fixed, and stained with a Diff-Quik staining kit (Dade, Dürdingen, Switzerland). The number of migrating T cells in five high-power fields (under 500x magnification; WILD Microscope, Heerbrugg, Switzerland) was evaluated. For each group, the results are expressed as the mean number of cells in one high-power field.

Spontaneous or cytokine-induced secretion of IL-8 or IL-1β from intestinal epithelial cell.

Test cells (HT-29 or Caco-2) were grown as confluent monolayers in 24-well tissue culture plates. After the cells reached confluence, the culture medium was changed and the cells (10^6 cells/well) were incubated for 24 h at 37°C with the IL-2 peptides (or control and derivative peptides) in 24-well plates without (spontaneous) or with the addition of TNF- α (TNF- α -induced). When TNF- α was used, the peptides were added to the cells 1 hour prior to the addition of TNF- α . Following culture, the supernatants were harvested and analyzed for IL-8 or IL-1 β secretion by ELISA using commercially available kits for IL-8 (Pharmingen) and IL-1 β (Genzyme), according to the manufacturers' protocols.

Purification of elastase and elastase digestion of IL-2. Neutrophils were isolated from the whole blood of a healthy donor by dextran sedimentation and Ficoll-Hypaque gradient centrifugation, as previously described (Yavin et al., 1996). Elastase was isolated by aprotinin-Sepharose affinity chromatography, followed by carboxymethyl-cellulose ion exchange chromatography, as developed by Baugh and Travis (Yavin et al., 1996; Baugh and Travis, 1976). The purified elastase, which was lyophilized and stored at -20°C until used, was biochemically checked to be entirely free from cross-contamination with cathepsin G (not shown). IL-2 was dissolved in distilled water to yield a 1mg/ml solution. Lyophilized neutrophil elastase (50 μ g) was dissolved in 1 ml of PBS and immediately added to the IL-2 solution. The elastase-IL-2 mixture was incubated (12 h) at 37°C. Aliquots were removed and stored at -20°C until subjected to HPLC separation.

Reverse phase HPLC. Elastase digests of IL-2 were purified with a prepacked Lichrospher-100 RP-18 column (4x25 mm, 5 μ m bead size), using a binary gradient formed with 0.1% trifluoroacetic acid (TFA) in H₂O (solution A) and 0.1% TFA in 75% acetonitrile in H₂O [(solution B) at $t=0$ min, B=3.5%, at $t=5$ min, B=3.5%, and then, the concentrations began to increase: at $t=60$ min, B=100% (i.e. 75% acetonitrile)]. The flow-rate was constant on 0.8ml/min. A Spectra-Physics SP8800 liquid chromatography system (Fremont, CA) equipped with an Applied Biosystems 757 (Foster City, CA) variable wavelength absorbency detector was used. The column effluents were monitored by UV absorbency at 220 nm, and the chromatograms were recorded on a ChromeJet integrator. Fractions that were 20% or more above valley levels were pooled, rotoevaporated to a minimal volume, and diluted with

HPLC grade water. The rotoevaporation and dilution with water step was performed twice to remove residual TFA and acetonitrile.

Amino acid composition of the synthetic peptides and amino acid sequence analysis. Purified peptide solutions [≈ 40 μg of peptide in 40 μl , with 5 μg of norvaline (an unnatural amino acid) as an internal standard] were rotoevaporated, hydrolyzed (10°C, 22 h) in 6N HCl under vacuum, and analyzed with an amino acid analyzer (HP1090, Palo Alto, CA). An on-line pre-column ortho-phthalaldehyde (OPA)/9-fluorenylmethoxycarbonyl (F-moc) derivatization, combined with reverse phase chromatography, was used to determine the amino acid composition of the peptides and the total peptide yield. Without exception, all of the peptides yielded excellent analysis ratios of corresponding amino acids deviations from expected values of less than 10%. Analysis of the elastase-generated IL-2 fractions was performed using a Model 470A gas phase microsequencer. Phenylthiohydantoin amino acid derivatives were separated on-line by reverse phase HPLC on a PTH C-18 column (2.1 x 220 mm) using a Model 120A analyzer (Applied Biosystems, CA).

Solid phase peptide synthesis. IL-2-derived peptides were prepared by conventional solid phase peptide synthesis, using an AMS-422 automated solid phase multiple peptide synthesizer (Abimed, Langenfeld, Germany). The Fmoc strategy was used for peptide chain assembly, according to the commercial protocol. In each reaction vessel, we used 12.5 μmol of Wang resin containing the first covalently bound corresponding N-Fmoc C-terminal amino acid (typically, polymer loadings of 0.3-0.7 mmol/g resin were used). Fmoc deprotection was achieved by two consecutive treatments with 20% piperidine in dimethyl formamide, usually 10-15 each min at 22°C, depending on the length of peptide and the Fmoc-protected amino acid type. The protecting groups used for the side chain of the amino acids were tert-butyloxycarbonyl for Trp and Lys; trityl for Asn, Cys, Gln and His; tert-butylester for Asp and Glu; and tert-butylether for Ser, Tyr and Thr. Usually, coupling was achieved using two successive reactions (typically 20-45 min each at 22°C, depending on the length of peptide and amino acid derivative type) with 50 μmol (4 eqv) of N-Fmoc-protected amino acid, 50 μmol (4 eqv) of benzotriazole-1-yl-oxy-tris- pyroolidino- phosphonium hexafluorophosphate (PyBop) reagent, and 100 μmol (8 eqv) of N-methylmorpholine were all dissolved in dimethylformamide (DMF). The peptide was cleaved from the polymer by reacting (2 h,

22°C) the resin with trifluoroacetic acid/H₂O/triethylsilane (90/5/5; v/v/v). The solution containing the crude, unprotected peptides was then cooled down to 4°C, precipitated with ether (4°C), and centrifuged (15 min, 3000 rpm, 4°C). The pellet was washed and centrifuged (x3) with ether, dissolved in 30% acetonitrile in H₂O, and lyophilized. The lyophilized material was reconstituted in double distilled water before use; only the stock solution, not the diluted material, was stored at -20°C.

Synthesis of cyclic peptides. The linear peptides **pep12**, **pep14**, **pep35**, **pep46** were cyclized as follows: 0.5-1.0 mg of SH-free peptide was dissolved in 1 ml of ammonium acetate buffer (0.1N, pH 7.0). A solution of K₃ [Fe(CN)₆] (1-5mM) in the same buffer was slowly added to the stirred peptide solution at room temperature, in a titrimetric fashion until a stable faint yellowish color was apparent. The solution revealed a negative 5,5'-dithio-bis(2-nitrobenzoic acid (DTNB; Ellman's Reagent)-test for free SH. Lyophilization and purification by HPLC were then performed as usual.

Staining of actin cytoskeleton. T cells were incubated (18 h, 37°C, 7.5% CO₂, humidified atmosphere) in culture medium. IL-2 or PMA was added to the cell cultures, which were then incubated for 24 h. The T cells were then washed and seeded onto FN-covered coverslips in the presence of either PMA (50 ng/ml), IL-2 (100 U/ml), or IL-2 peptides (0.1 ng/ml). After 1 h at 37°C, the adherent cells were fixed (3 min) with paraformaldehyde (3%) and Triton X-100 (0.5%), washed, and fixed (20 min) again with paraformaldehyde (3%). The fixed adherent cells were washed, treated with TRITC-phalloidin, and washed again. Photographs (x1000 magnification) were then taken.

Example 1

Induction of the adhesion of T cells to ECM, FN, LN, and CO-IV by IL-2. We examined the ability of soluble IL-2 to induce adhesion of human T cells to ECM, FN, LN, and CO-IV. The results indicated that IL-2 induced T cell adhesion to FN, LN, and CO-IV (Fig. 1A), as well as to intact ECM (Fig. 1B). Note that the adhesion of T cells to LN induced by IL-2 was lower than that induced to the other ECM glycoproteins. When T cells were activated only with PMA, 45±4.4% of them adhered to immobilized ECM and ECM glycoproteins (not shown). IL-2-induced T cell adhesion to the ECM glycoproteins was inhibited by anti-human

β_1 integrin mAbs (not shown), which suggests that the pro-adhesive effects of IL-2 were induced via cell surface-expressed integrins. However, under our experimental conditions, IL-2 did not alter the T cell surface expression of β_1 integrins (not shown). Thus IL-2, in addition to other pro-inflammatory mediators, appears to regulate the adhesiveness of resting human T cells to immobilized ECM and ECM glycoproteins.

Example 2

The effects of IL-2 fragments obtained by elastase degradation on the interaction of T cells with FN. We have assumed that the degradation of IL-2 can occur in the inflamed milieu in which both cytokines, such as IL-2, and proteolytic enzymes, such as neutrophil elastase, are present. We also hypothesized that, in contrast to the intact IL-2 molecule, certain portions of IL-2 can abrogate the adhesiveness of activated T cells to ECM ligands. Hence, elastase and soluble IL-2 were incubated together at physiologic conditions. HPLC analysis of the elastase-degraded IL-2 revealed at least 8 peaks of IL-2, each of which represented at least one low molecular weight protein fragment (Fig. 2).

Next, we examined the ability of the HPLC-purified IL-2 fractions, which were generated by elastase-degradation, to inhibit soluble IL-2-induced interactions of T cells with FN. We chose to investigate the major peaks of HPLC-purified, elastase-degraded fractions of IL-2. Fractions 2, 7, and 8 inhibited the adhesion of T cells to immobilized FN in a dose-dependent and statistically significant fashion, whereas fractions 4 and the HPLC buffer did not (Fig. 3). Thus, certain IL-2 fragments, obtained by neutrophil elastase-processing of the cytokine, can inhibit IL-2-induced adhesion of T cells to FN.

Example 3

The effect of synthetic peptides, with putative amino acid compositions corresponding to fractions 2 and 8, on IL-2-induced T cell adhesion to FN. Next, the primary sequence of fractions 2 and 8 were analyzed by gas phase chromatography, because these elastase-generated fractions of IL-2 appeared to contain adhesion-suppressive peptides. Our analysis revealed that fragment 2 contained an Ile-Val-Leu (IVL; **pep1**; IL-2₁₁₂₋₁₁₄) and an Arg-Met-Leu-Thr (RMLT; **pep2**; IL-2₅₈₋₆₁) peptide, whereas fragment 8 contained a

Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (EFLNRWIT; **pep3**; IL-2₁₃₆₋₁₄₃) octa-peptide. These three peptides were synthesized, and their effects on IL-2-induced T cell adhesion to FN were studied. The Ile-Val-Leu, Arg-Met-Leu-Thr, and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr, inhibited, in a dose-dependent manner, the adhesion of IL-2-activated T cells to FN; the Arg-Met-Leu-Thr is apparently the most potent inhibitor (Fig. 4A). Maximum inhibition was achieved with about 0.1 pg/ml (0.2 pM) for Arg-Met-Leu-Thr, and 1 pg/ml of either Ile-Val-Leu or Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (2.91 and 0.92 pM, respectively). The inhibitory dose-response curves of Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr are similar to those of the HPLC fractions 2 and 8, respectively, from which they were derived.

To examine the specificity, on the biologic and chemical levels, of the inhibition of T cell adhesion by the elastase-generated, synthetic IL-2 peptides, we synthesized the three IL-2 peptides in their inverse amino acid sequences, Leu-Val-Ile, Thr-Leu-Met-Arg, and Thr-Ile-Trp-arg-Asn-Leu-Phe-Glu, and then tested their effects on IL-2-induced T cell adhesion to FN. The results, shown in Fig. 4B, indicate that none of these peptides, tested in a broad range of dosages, interfere with T cell adhesion. Thus, the anti-adhesive effects of Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr peptides of IL-2 appear to be due to their direct biologic effect on responding lymphocytes.

Do the IL-2 peptides, Ile-Val-Leu, Arg-Met-Leu-Thr, and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr have to be present during the entire period of the assay to exert their inhibitory effects? The results, shown in Fig. 4C, indicate that most of the anti-adhesive effects of the three peptides persevered even if these peptides (at 1 pg/ml) were removed from the T cells prior to their activation with IL-2 and seeding onto the FN-coated surfaces. Apparently, their prolonged inhibitory potential may involve active intracellular signaling pathways. These results suggest that the IL-2 peptides neither exert their inhibitory activities on T cell adhesion to FN via binding to the ECM protein, nor to FN-specific β_1 integrins expressed on the adhering T cells.

Example 4

Ile-Val-Leu, Arg-Met-Leu-Thr and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr inhibit T cell adhesion to LN, CO-IV and FN. The next experiment was designed and performed to verify that the

three IL-2-derived peptides indeed affect T cell interactions with ECM glycoproteins other than FN. T cells were pre-exposed to the three peptides (at 10 pg/ml), and then activated with IL-2. The treated cells were then added to microtiter wells coated with CO-IV, LN, and FN. The results, shown in Fig. 5, indicate that both Ile-Val-Leu, Arg-Met-Leu-Thr, and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr inhibit T cell adhesion to the three major cell-adhesive glycoproteins of the ECM (Fig. 5), suggesting that the elastase-generated IL-2 peptides exert their inhibitory effects over different subsets of β_1 integrins.

Example 5

Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr inhibit T cell chemotactic migration induced by IL-2 or MIP-1 β . Immune cell migration is the outcome of a subtle biological equilibrium existing between adhesion and detachment events. Lymphocyte adhesion to the subendothelial ECM and subsequent migration are two active processes that can overlap, but are not mutually dependent events. Adhesion and migration may depend on the ability of the T cells to continuously integrate different pro- and anti-adhesive signals via their versatile receptors for ECM, chemokines, cytokines, and possibly, also antigenic moieties. Therefore, we next examined the effects of the Ile-Val-Leu, Arg-Met-Leu-Thr, and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr peptides (at 1 pg/ml) on the IL-2- and MIP-1 β -induced T cell chemotaxis through FN-coated polycarbonate membranes. The gradient generated by MIP-1 β and IL-2, which were placed in the lower compartment of the 48-well chemotaxis apparatus, induced a marked T cell migration through FN-coated membranes, which was about 3 to 4-fold higher than the control (Fig. 6). Both Ile-Val-Leu, Arg-Met-Leu-Thr, and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr markedly ($p < 0.01$) inhibited T cell migration toward IL-2, by about 30, 90, and 60%, respectively. However, although the Ile-Val-Leu peptide, and to a lesser degree also the Arg-Met-Leu-Thr peptide, markedly inhibited (80% and 60%, respectively) T cell chemotaxis toward MIP-1 β , the Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr showed only a limited inhibitory effect on the chemokine-induced T cell chemotaxis. Thus, in addition to the capacity of the Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr peptides to inhibit T cell-ECM adhesion, they seem to inhibit T cell migration through FN in response to a diffusible gradient produced by IL-2 or MIP-1 β .

Example 6

Inhibition by the Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr peptides of T cell adhesion to FN induced by various pro-inflammatory mediators and by mAb specific for molecules expressed on T cells. The preceding chemotaxis experiments indicated that the

5 Ile-Val-Leu and Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr peptides can inhibit T cell adhesion and migration through FN barriers induced not only by IL-2, but also by the chemokine MIP-1 β . Therefore, in an attempt to further understand the possible physiologic relevance of such phenomena, we examined the ability of these peptides to inhibit the adhesion to FN of T cells stimulated by modes other than IL-2. At 10 pg/ml, both Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr

10 and Ile-Val-Leu inhibited T cell adhesion to FN that was induced by various stimulators of T cells and modulators of the β_1 integrin functions tested (Fig. 7). However, at 0.1 pg/ml, Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr did not inhibit the PMA- and 8A2-induced adhesion, and the Ile-Val-Leu peptide did not inhibit PMA-, 8A2-, and anti-CD3 mAb-induced T cell adhesion to FN, which indicates that these modes of activation are less susceptible to

15 IL-2-derived peptide-induced suppression than IL-2-mediated activation. In experiments similar to those shown in Figs. 7A and 7B, the control peptides (corresponding inverse sequences) did not affect T cell adhesion to FN induced by the indicated activators (data not shown). Hence, Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr and Ile-Val-Leu apparently inhibit T cell adhesion to the FN component of ECM via a common intracellular event that is linked to the

20 regulation of the avidities and affinities of β_1 integrins, and therefore, to their ligand recognition and binding.

Example 7

Inhibition of the reorganization of the actin cytoskeleton in FN-attached activated T cells by the Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr peptide. The adhesion of immune cells to ECM is

25 dependent on the sequestering of the cytoplasmic domains of integrins in focal adhesion sites, together with actin-containing microfilament bundles (Sanchez-Mateos et al., 1996). Therefore, we examined the effect of Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr on the morphologies of adherent T cells. The T cells were activated with IL-2 or PMA, treated with the IL-2

30 peptides, and seeded onto FN-coated coverslips. After incubation and fixation, the actin cytoskeleton of attached T cells was stained with TRITC-conjugated phalloidin. The morphologies of FN-bound IL-2- and PMA-activated T cells (Figs. 8A, 8B) were markedly

different from those of non-activated lymphocytes (Fig. 8E); the activated T cells appeared spread, and their actin cytoskeleton performed distinct structures typical of ECM-adherent cells. The Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr peptide inhibited the redistribution of the actin skeleton in both the IL-2- (Fig. 8C) and PMA-treated (Fig. 8D) FN-adherent T cells. Control peptides (reverse sequences of each peptide **pep1**, **pep2**, **pep3**) did not inhibit the actin reorganization of the activated T cells (not shown). Hence, the adhesion-inhibiting activity of the IL-2-derived peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr, similar to the Ile-Val-Leu peptide (data not shown), appears to involve inhibition of the redistribution of the actin cytoskeleton, and therefore, changes in cell shape and spreading.

Example 8

The effect of synthetic IL-2 derived peptides and derivatives thereof on spontaneous cytokine (IL-8) secretion from intestinal epithelial cells. Human colonic epithelial cell lines, such as HT-29, have been shown to secrete pro-inflammatory cytokines such as IL-1 β and IL-8, in response to cytokines such as TNF- α , IL-1 β , IFN- γ , and to LPS (Ecknamm et al., 1993), or as a response to bacterial invasion (Jung et al., 1995).

The effect of the synthetic IL-2 peptides **pep1**, **pep2**, **pep3**, and other synthetic derivatives of the sequences shown in Table 1 below, was tested on spontaneous IL-8 secretion from HT-29 cells as follows: HT-29 cells (10^6 cells/well) were seeded and incubated in culture medium for 24 h at 37°C in 24-well plates with the desired peptide (1 nM), the supernatants were collected and IL-8 levels were determined by an ELISA assay, using Pharmingen antibody pairs, according to the manufacturer's instructions. The results in Table 1 show the O.D. readings of the ELISA assay (at 405 nm) as well as the percentage of inhibition of IL-8 secretion. A level of inhibition of about 30% or higher indicates that the peptide has relevant anti-inflammatory activity. These results show that the peptides inhibit the spontaneous secretion of IL-8 from a human colonic epithelial cell line and are, therefore, good candidates for the treatment of inflammatory, particularly, bowel diseases.

Table 1: Inhibitory effect of pep1, pep2, pep3 and analogues thereof on spontaneous IL-8 secretion from intestinal epithelial cells

<u>Peptide</u>	<u>IL-8 secretion</u>	
	<u>Average O.D.</u>	<u>% inhibition</u>
Background	71±1.4	
Medium alone	488±8.5	0
(pep1) Ile-Val-Leu,	54±19.1	104
(pep4) Asn-Ile-Asn-Val-Ile-Val-Leu,	34±3.5	109
(pep5) Ile-Val-Leu-Glu-Leu-Lys-Gly,	35±0.7	109
(pep6) Asn-Val-Ile-Val-Leu	100±14.8	93
(pep7) Ala-Val-Leu	170±8.5	76
(pep8) Ile-Ala-Leu	35±4.9	109
(pep9) Ile-Val-Ala	100±9.9	93
(pep10) Glu-Val-Leu	125±19.1	87
(pep2) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr	36±4.2	108
(pep15) Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr	33±0.7	109
(pep16) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys	126±7.1	87
(pep17) Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr	150±6.4	81
(pep18) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser	162±12.0	78
(pep19) Leu-Asn-Arg-Trp-Ile-Thr	152±30.4	81
(pep20) Arg-Trp-Ile-Thr	191±4.9	71

(pep21) Glu-Phe-Leu-Asn	195±14.1	70
(pep22) Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr	158±4.2	79
(pep23) Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr	36±14.1	108
(pep24) Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr	187±16.3	72
(pep25) Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr	172±2.8	76
(pep26) Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr	156±27.6	80
(pep27) Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr	309±18.4	43
(pep28) Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr	120±8.5	88
(pep29) Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr	139±19.8	84
(pep30) Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr	123±9.2	88
(pep31) Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr	112±18.4	90
(pep32) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala	80±24.0	98
(pep33) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr- NH ₂	73±5.7	100
(pep3) Arg-Met-Leu-Thr	44±0.7	107
(pep36) Ala-Met-Leu-Thr	201±17.7	69
(pep37) Arg-Ala-Leu-Thr	222±24.0	64
(pep38) Arg-Met-Ala-Thr	157±1.4	79
(pep39) Arg-Met-Leu-Ala	160±21.9	79
(pep40) Lys-Met-Leu-Thr	179±17.7	74
(pep41) Arg-Val-Leu-Thr	132±17.0	85
(pep42) Arg-Met-Leu-Thr-NH ₂	226±28.3	63
(pep43)	57±12.7	103

Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr		
(pep44)	307±29.7	43
Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr		

The effect of the peptides was also tested on the TNF- α induced secretion of IL-1 β from intestinal epithelial cells. Test cells (HT-29 or Caco-2) were grown as confluent monolayers in 24-well tissue culture plates. After the cells reached confluence, the culture medium was changed and the cells (10^6 cells/well) were incubated for 24 h at 37°C with the IL-2 peptides (or control and derivative peptides) in 24-well plates with the addition of TNF- α . The peptides were added to the cells 1 hour prior to the addition of TNF- α . Following culture, the supernatants were harvested and analyzed for IL-1 β secretion by ELISA using a commercially available kit (Genzyme), according to the manufacturers' protocols. The results are similar to those obtained above, the peptides being shown to inhibit TNF- α induced secretion of IL-1 β in similar levels as above (data not shown).

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CLAIMS

1. A synthetic anti-inflammatory peptide derived from IL-2, and anti-inflammatory derivatives of said peptide.
2. A synthetic anti-inflammatory peptide or peptide derivative according to claim 1, which inhibits at least one of the following processes in vitro: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine, from intestinal epithelial cells.
3. A synthetic anti-inflammatory peptide or peptide derivative according to claim 1 or 2, which inhibits in vitro: (i) adhesion of activated T cells to fibronectin, laminin and/or collagen-type IV; (ii) chemotactic migration of T cells through fibronectin; and/or (iii) spontaneous or TNF- α -induced secretion of IL-8 or IL-1 β , from intestinal epithelial cells.
4. A synthetic peptide according to any one of claims 1 to 3, selected from:
- (i) peptides **pep1**, **pep2**, and **pep3** of the sequences:
 - (**pep1**) Ile-Val-Leu
 - (**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr
 - (**pep3**) Arg-Met-Leu-Thr
 - (ii) peptides obtained from **pep2** by deletion of one or more amino acid residues;
 - (iii) peptides obtained by addition to peptides (i) or (ii) of one or more natural or non-natural amino acid residues;
 - (iv) peptides obtained by replacement of one or more amino acid residues of peptides (i) to (iii) by the corresponding D-stereomer, by another natural amino acid residue or by a non-natural amino acid residue;
 - (v) chemical derivatives of the peptides (i) to (iv);
 - (vi) cyclic derivatives of peptides (i) to (v);
 - (vii) dual peptides consisting of two of the same or different peptides (i) to (vi),

wherein the peptides are covalently linked to one another directly or through a spacer; and

(viii) multimers comprising a number of the same or different peptides (i) to (vi).

5 5. The synthetic peptide Ile-Val-Leu (**pep1**) and derivatives thereof according to claim 4, obtained by:

(a) elongation by up to 3-4 further amino acid residues at the N- and/or C-terminal, preferably according to the natural sequence of IL-2;

10 (b) substitution of the Ile residue by a natural or non-natural amino acid hydrophilic polar neutral or negatively charged, or hydrophobic non-polar neutral amino acid residue, preferably selected from Glu, Asp, Asn, Gln, Ala, Val;

(c) substitution of the Val residue by a hydrophobic, non-charged natural or non-natural amino acid residue, preferably selected from Ala, Ile, Leu, Met, Nle, Phe;

15 (d) substitution of the Leu residue by a hydrophobic, non-charged natural or non-natural amino acid residue, preferably selected from Ala, Ile, Met, Nle, Phe, Val;

(e) amidation of the C-terminal Leu residue,

(f) cyclization of **pep1** or of any peptide of (a) to (e); and

(g) any combination of (a) to (f).

20 6. A synthetic peptide according to claim 5, selected from:

(**pep1**) Ile-Val-Leu

(**pep4**) Asn-Ile-Asn-Val-Ile-Val-Leu,

(**pep5**) Ile-Val-Leu-Glu-Leu-Lys-Gly,

(**pep6**) Asn-Val-Ile-Val-Leu

25 (**pep7**) Ala-Val-Leu

(**pep8**) Ile-Ala-Leu

(**pep9**) Ile-Val-Ala

(**pep10**) Glu-Val-Leu

(**pep11**, linear) and (**pep12**, cyclic) Cys-Ile-Val-Leu-Ala-Cys and,

30 (**pep13**, linear) and (**pep14**, cyclic) Cys-Ile-Val-Leu-Ala-Ala-Cys

7. The synthetic peptide Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr (**pep2**), and derivatives

thereof according to claim 4, obtained by:

(a) elongation by up to 4 further amino acid residues at the C and/or N terminal ends, preferably according to the natural sequence of IL-2;

(b) substitution of the Glu residue by a natural or non-natural charged or polar charged amino acid residue, preferably selected from Lys, Arg, Asp, Gln, Asn;

(c) substitution of the Phe residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(d) substitution of the Leu residue by a natural or non-natural hydrophobic aliphatic or aromatic amino acid residue, preferably selected from Ala, Val, Ile, Leu, Tyr, Trp, Phe, Met, Nle;

(e) substitution of the important Asn residue by a hydrophilic, non-charged, aliphatic natural or non-natural amino acid residue such as Gln;

(f) substitution of the Arg residue by a positively charged, natural or non-natural amino acid residue, preferably selected from Lys, Orn, homoArg;

(g) substitution of the Trp residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Ile, Leu, Nle, Tic, Phe, 4-phenyl-Phe, 4-methyl-Phe;

(h) substitution of the Ile residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Tyr, Phe, Leu, Nle, Tic;

(i) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue preferably selected from Cys, Ser;

(j) truncation by up to 4 amino acid residues from either the C or N terminal;

(k) amidation of the C-terminal Thr;

(l) cyclization of **pep2** or of any peptide of (a) to (k); and

(m) any combination of (a) to (l).

8. A peptide according to claim 7, selected from:

(**pep2**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr

(**pep15**) Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr

(**pep16**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys

- (**pep17**) Ala-Thr-Ile-Val-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr
 (**pep18**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Phe-Cys-Gln-Ser
 (**pep19**) Leu-Asn-Arg-Trp-Ile-Thr
 (**pep20**) Arg-Trp-Ile-Thr
 5 (**pep21**) Glu-Phe-Leu-Asn
 (**pep22**) Ala-Phe-Leu-Asn-Arg-Trp-Ile-Thr
 (**pep23**) Lys-Phe-Leu-Asn-Arg-Trp-Ile-Thr
 (**pep24**) Glu-Ala-Leu-Asn-Arg-Trp-Ile-Thr
 (**pep25**) Glu-Val-Leu-Asn-Arg-Trp-Ile-Thr
 10 (**pep26**) Glu-Phe-Ala-Asn-Arg-Trp-Ile-Thr
 (**pep27**) Glu-Phe-Leu-Ala-Arg-Trp-Ile-Thr
 (**pep28**) Glu-Phe-Leu-Asn-Ala-Trp-Ile-Thr
 (**pep29**) Glu-Phe-Leu-Asn-Glu-Trp-Ile-Thr
 (**pep30**) Glu-Phe-Leu-Asn-Arg-Ala-Ile-Thr
 15 (**pep31**) Glu-Phe-Leu-Asn-Arg-Trp-Ala-Thr
 (**pep32**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Ala
 (**pep33**) Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-NH₂ and,
 (**pep34**, linear) and (**pep35**, cyclic)
 Cys-Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr-Ala-Cys.

20 9. The synthetic peptide Arg-Met-Leu-Thr (**pep3**), and derivatives thereof according to claim 4, obtained by:

- (a) elongation by up to 4 further amino acid residues at the C and/or N terminal end, preferably according to the natural sequence of IL-2;
 25 (b) substitution of the Arg residue by a natural or non-natural positively charged amino acid residue, preferably selected from Lys, Orn, homoArg, diaminobutyric acid;
 (c) substitution of the Met residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Ile, Leu, Nle, Tic;
 (d) substitution of the Leu residue by a natural or non-natural hydrophobic, aliphatic or aromatic, amino acid residue, preferably selected from Phe, Tyr, Nle, Tic;
 30 (e) substitution of the Thr residue by an aliphatic hydrophobic amino acid residue such as Ala, Ile, Leu, or a hydroxy- or thio-containing amino acid residue such as Ser,

Cys;

(f) amidation of the C-terminal Thr residue;

(g) cyclization of **pep3** or of any peptide of (a) to (f); and

(h) any combination of (a) to (g).

5

10. A peptide according to claim 9, selected from:

(**pep3**) Arg-Met-Leu-Thr

(**pep36**) Ala-Met-Leu-Thr

(**pep37**) Arg-Ala-Leu-Thr

10

(**pep38**) Arg-Met-Ala-Thr

(**pep39**) Arg-Met-Leu-Ala

(**pep40**) Lys-Met-Leu-Thr

(**pep41**) Arg-Val-Leu-Thr

(**pep42**) Arg-Met-Leu-Thr-NH₂

15

(**pep43**) Pro-Lys-Leu-Thr-Arg-Met-Leu-Thr

(**pep44**) Arg-Met-Leu-Thr-Phe-Lys-Phe-Tyr and,

(**pep45**, linear) and (**pep46**, cyclic) Cys-Arg-Met-Leu-Thr-Ala-Cys.

11. A method of selecting anti-inflammatory peptides derived from pro-inflammatory
IL-2, which comprises:

20

(i) carrying out enzymatic digestion of IL-2 with a proteolytic enzyme that participates in the breakdown of the extracellular matrix (ECM);

(ii) testing the fractions obtained in (i) for their in vitro ability to inhibit at least one of the following processes: (a) adhesion of activated T cells to ECM proteins; (b) chemotactic migration of T cells through ECM proteins; (c) cytokine- or mitogen-induced T cell proliferation; (d) cytokine secretion by cytokine- or mitogen-stimulated T cells; (e) spontaneous or cytokine-induced secretion of a cytokine, e.g. IL-8 or IL-1 β , from intestinal epithelial cells,

25

(iii) selecting the fractions of (ii) active in at least one of the bioassays (a) to (e), fractionating each fraction to identify individual peptides thereof, and submitting each identified peptide to sequencing and synthesis;

30

(iv) carrying out one or more of the bioassays (a) to (e) with a synthetic

peptide identified in step (iii), and selecting those peptides that show significant inhibitory activity in at least one of the bioassays (a) to (e).

12. The method according to claim 11, wherein the proteolytic enzyme is elastase, a collagenase or a metalloprotease.

13. An anti-inflammatory peptide obtainable by a method of claim 11 or 12.

14. A pharmaceutical composition comprising at least one synthetic peptide or peptide derivative according to any one of claims 1 to 10, and a pharmaceutically acceptable carrier.

15. Use of a synthetic peptide as defined in any of claims 1 to 10 or 13, for the preparation of a pharmaceutical composition for the treatment of inflammatory disorders.

16. Use according to claim 15, wherein the inflammatory disorder is an acute or chronic inflammatory disorder.

17. Use according to claim 16, wherein the inflammatory disorder is an autoimmune disease selected from rheumatoid arthritis, diabetes type I, multiple sclerosis, systemic lupus erythematosus, uveitis, bowel inflammation and Crohn's disease.

18. A method for the treatment and/or alleviation of acute and chronic inflammatory disorders comprising administering to a subject in need thereof an effective amount of an anti-inflammatory synthetic peptide according to any one of claims 1-10.

ABSTRACT

- 5 Synthetic anti-inflammatory peptides derived from the sequence of IL-2 are provided. Parent peptides of the sequences Ile-Val-Leu, Glu-Phe-Leu-Asn-Arg-Trp-Ile-Thr and Arg-Met-Leu-Thr, were obtained by elastase enzymatic digestion of IL-2, synthesized and modified. The peptides are useful in conditions of acute and chronic inflammation such as in autoimmune diseases.

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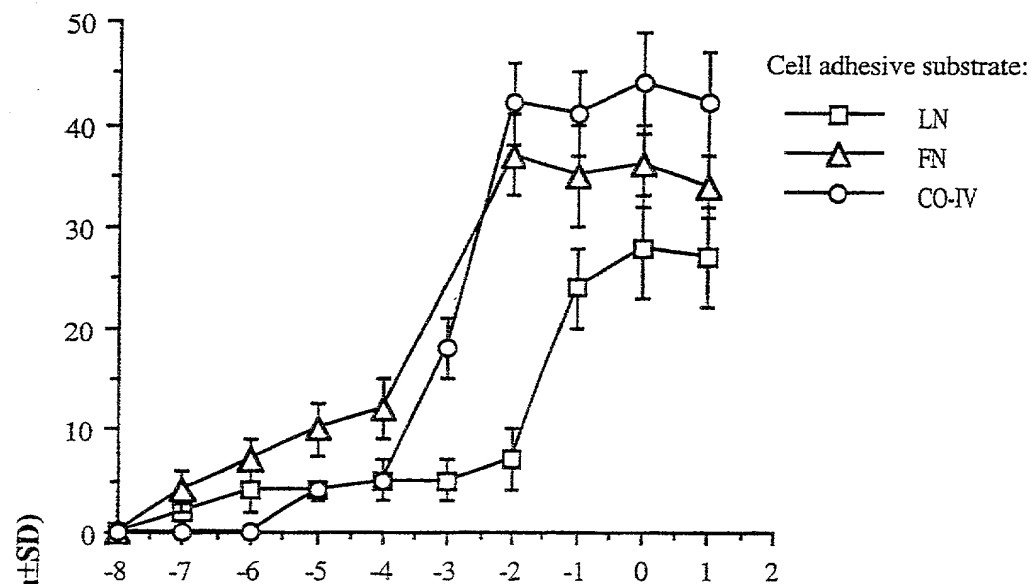


Fig. 1A

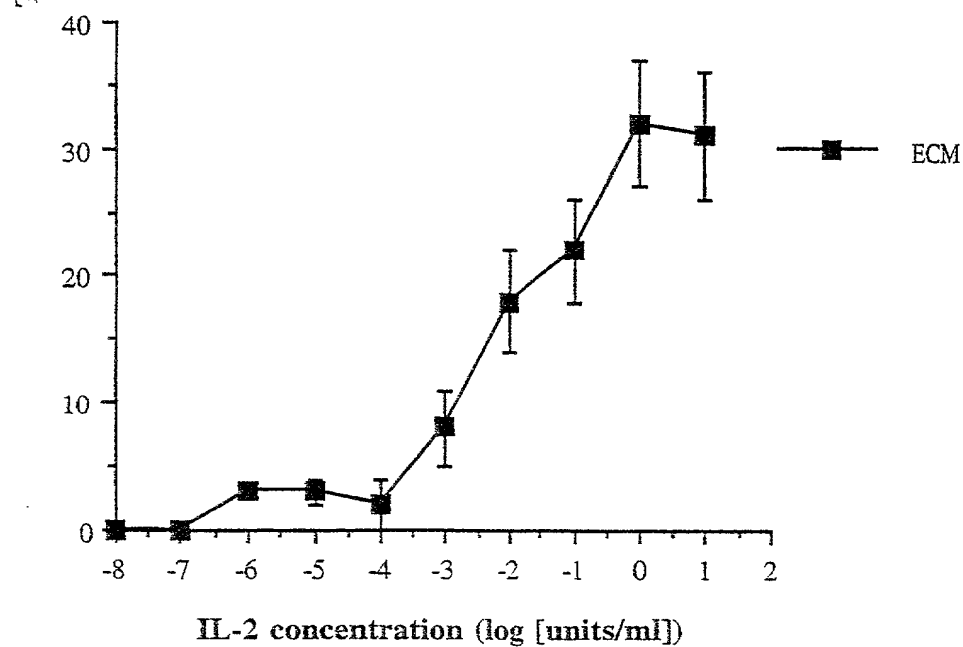


Fig. 1B

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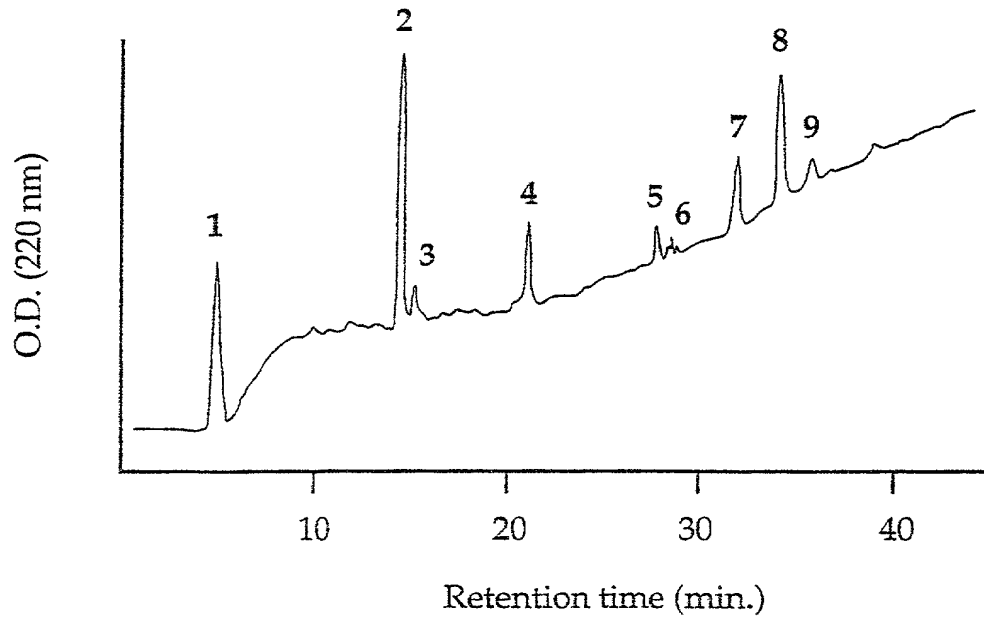


Fig. 2

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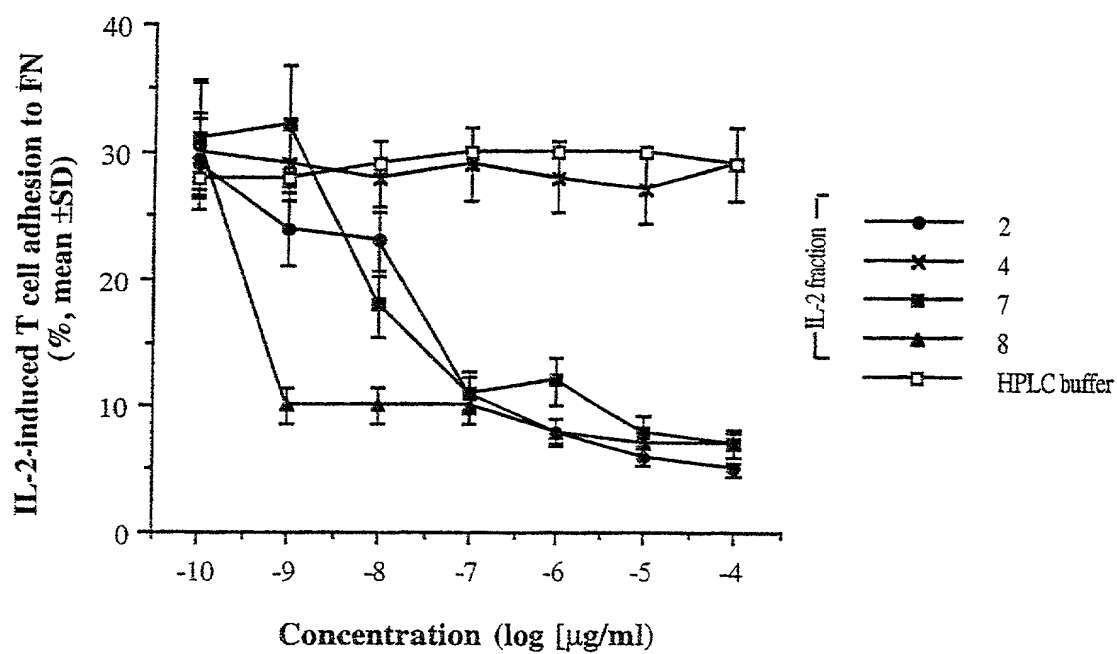


Fig. 3

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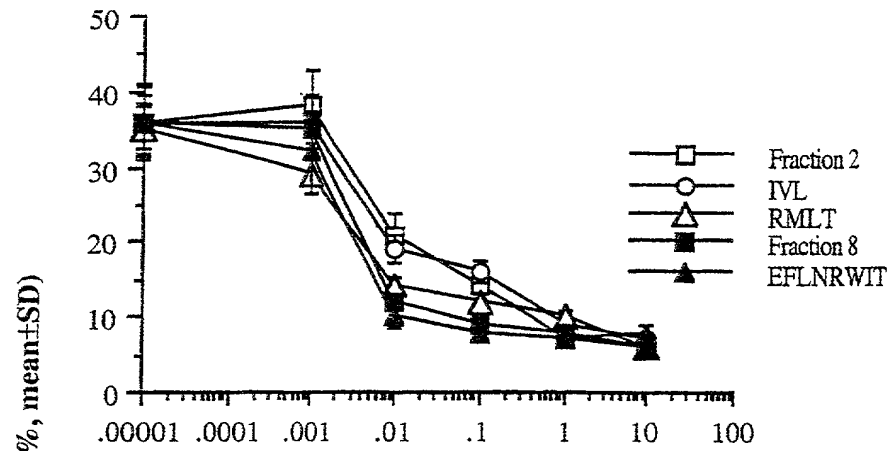


Fig 4A

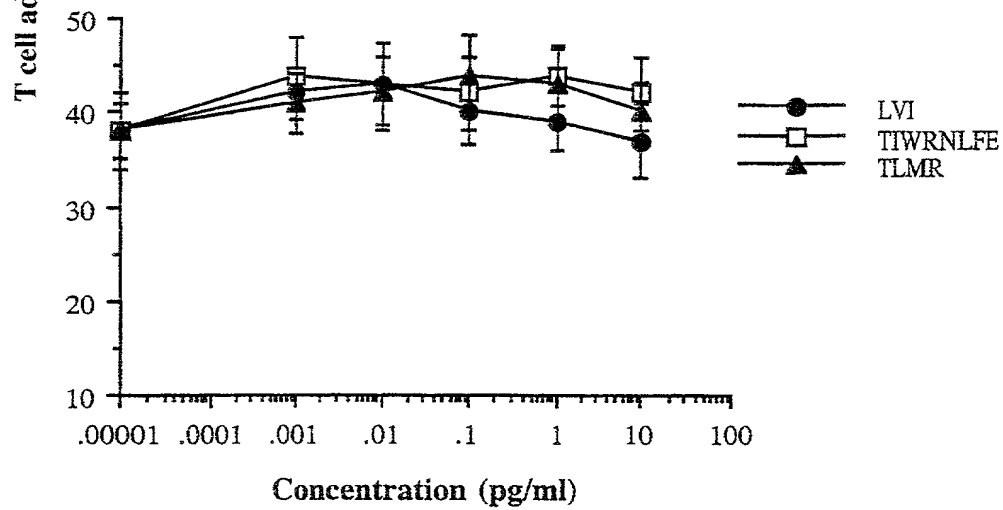


Fig. 4B

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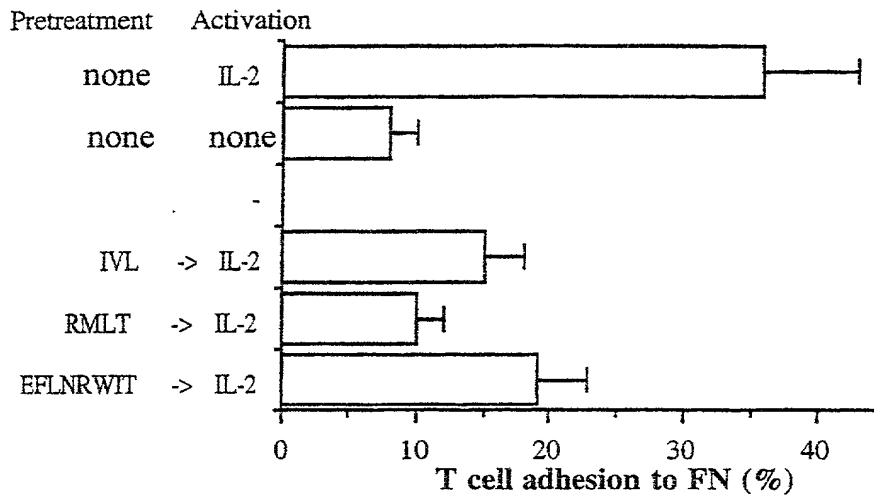


Fig. 4C

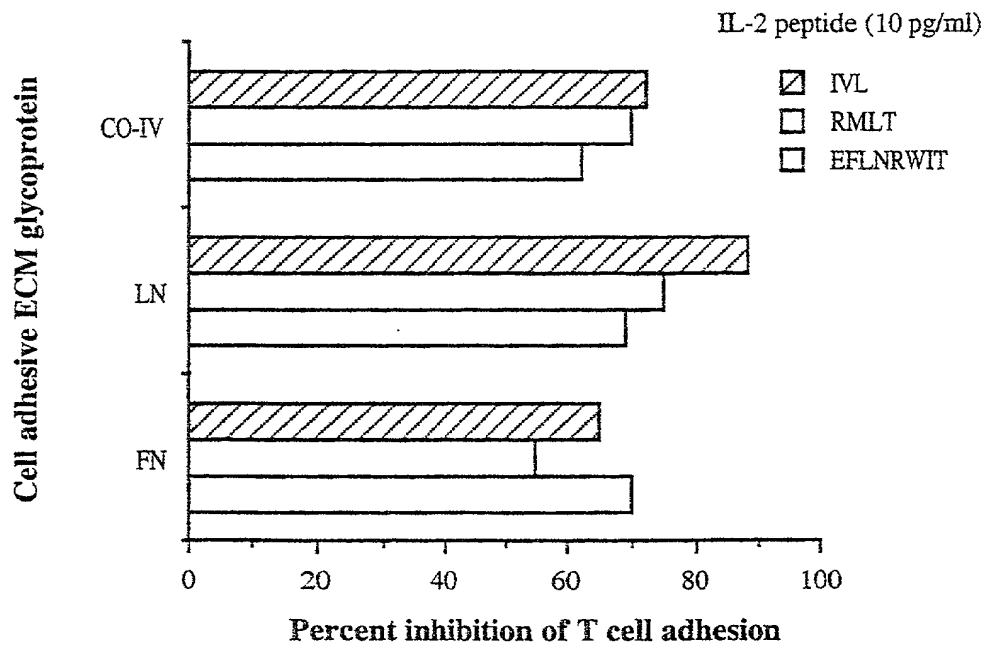


Fig. 5

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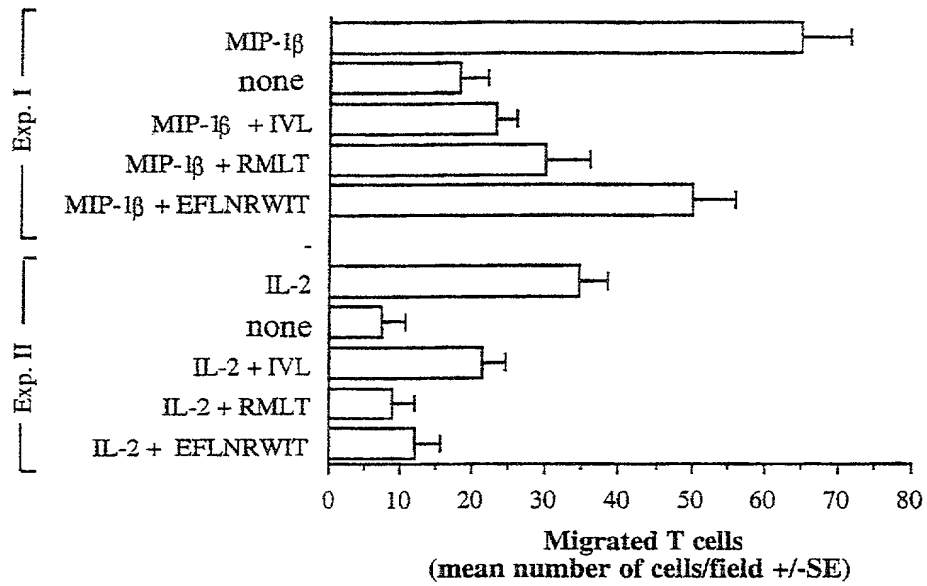


Fig. 6

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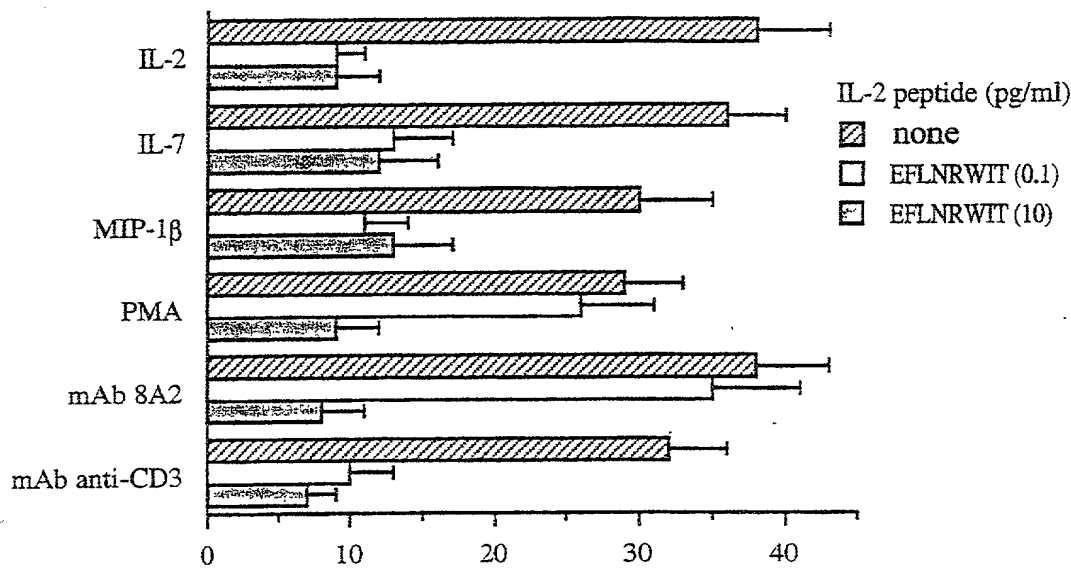


Fig. 7A

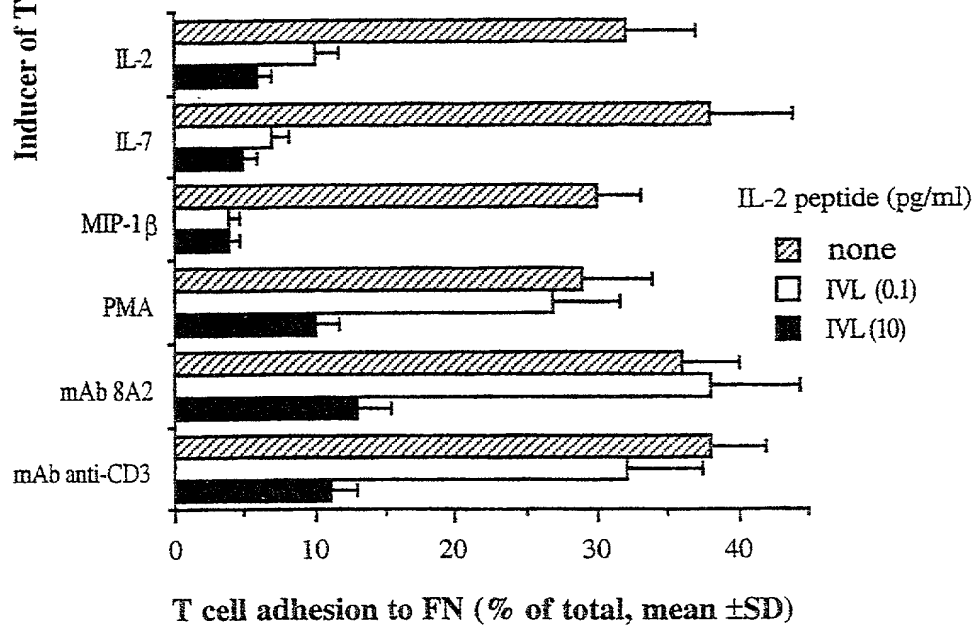


Fig. 7B

Fig. 8A

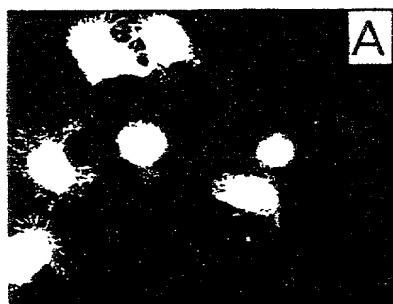


Fig. 8B



Fig. 8C



Fig. 8D

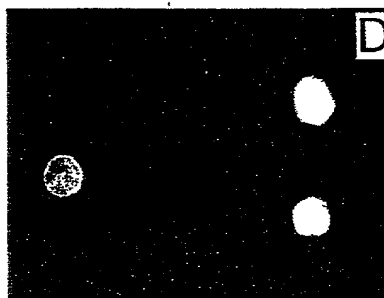
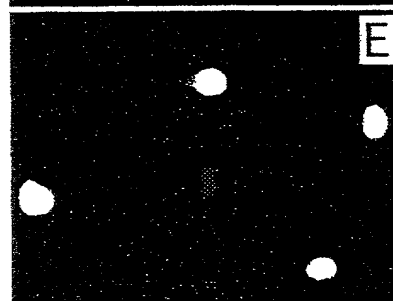


Fig. 8E



Combined Declaration for Patent Application and Power of Attorney

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

ANTI-INFLAMMATORY PEPTIDES DERIVED FROM IL-2 AND ANALOGUES THEREOF

the specification of which (check one)

- [] is attached hereto;
 [] was filed in the United States under 35 U.S.C. §111 on _____, as
 U.S. Appln. No. _____*; or
 [X] was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an
 international (PCT) application, PCT/IL99/00448; filed 19 August 1999, entry requested on
 _____*; national stage application received U.S. Appln. No. _____*, §371/§102(e)
 date _____ (* if known)

and was amended on _____ (if applicable).
(include dates of amendments under PCT Art. 19 and 34 if PCT)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119 and 365 of any prior foreign application(s) for patent or inventor's certificate, or prior PCT application(s) designating a country other than the U.S., listed below with the "Yes" box checked and have also identified below any such application having a filing date before that of the application on which priority is claimed

9818370.0	Great Britain	21 August 1998	[X]	[]
(Number)	(Country)	(Day Month Year Filed)	YES	NO
126009	Israel	31 August 1998	[X]	[]
(Number)	(Country)	(Day Month Year Filed)	YES	NO
129980	Israel	16 May 1999	[X]	[]
(Number)	(Country)	(Day Month Year Filed)	YES	NO

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or prior PCT application(s) designating the U.S. listed below, or under §119(e) of any prior U.S. provisional applications listed below, and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information as defined in 37 C.F.R. §1.56(a) which occurred between the filing date of the prior application and the national filing date of this application:

_____ (Application No.)	_____ (Day Month Year Filed)	_____ (Status: patented, pending, abandoned)
_____ (Application No.)	_____ (Day Month Year Filed)	_____ (Status: patented, pending, abandoned)
_____ (Application No.)	_____ (Day Month Year Filed)	_____ (Status: patented, pending, abandoned)

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

All of the practitioners associated with Customer Number 001444

Direct all correspondence to the address associated with **Customer Number 001444**; i.e.,

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The undersigned hereby authorizes the U.S. Attorneys or Agents appointed herein to accept and follow instructions from Yeda Research and Development Co. Ltd. as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

09/09/99 15:26:26

Title: ANTI-INFLAMMATORY PEPTIDES DERIVED FROM IL-2 AND ANALOGUES THEREOF

U.S. Application filed _____, Serial No. _____

PCT Application filed 19 August 1999, Serial No. PCT/IL99/00448

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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POST OFFICE ADDRESS <u>41 Moskovich Street, 76468 Rehovot, Israel</u>			
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FULL NAME OF SIXTH JOINT INVENTOR		INVENTOR'S SIGNATURE	DATE
RESIDENT		CITIZENSHIP	
POST OFFICE ADDRESS			
FULL NAME OF SEVENTH JOINT INVENTOR		INVENTOR'S SIGNATURE	DATE
RESIDENT		CITIZENSHIP	
POST OFFICE ADDRESS			

SEQUENCE LISTING

<110> Yeda Research and Development Co. Ltd.

<120> Anti-inflammatory peptides derived from IL-2 and analogues thereof

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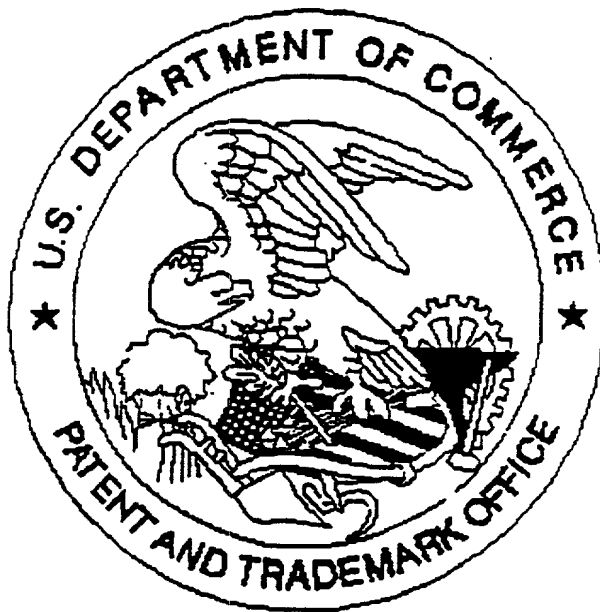
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Office of Initial Patent Examination -- Scanning Division



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for scanning. (Document title)

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for scanning. (Document title)

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